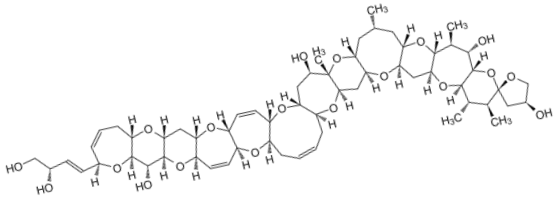
Ciguatoxin1B ELISA Kit



"CTX-ELISA™1B" Operation Manual

CSI, Code No. 8100

Wako, Code No. 382-14341

This kit contains sufficient reagents for 2 x 96 determinations.

Code	Components	Quantity
8111	Plate Wash Buffer x20 conc, **W-1**	1 x 50 mL
8112	STD/Sample Diluent, **D-1**	1 x 50 mL
8113	Anti-CTX1B-ALP Diluent, **D-2**	1 x 50 mL
8114	ALP Substrate Solution (pNPP), **R-1**	1 x 50 mL
8115	ELISA Assay Plate CTX1B	2 x 96 well
8116	Anti-CTX1B-ALP	1 x 250 µL
8117	CTX-1B STD 5 ng/mL *1	1 x 100 µL
--	Adhesive plastic sheet	6 sheet
--	Operation manual	1

Storage : Keep in the refrigerator (2~8˚C) promptly upon receipt, do not store in freezer.
Expiration date : Indicated on the box.

*1 The standard solution of Ciguatoxin1B "**CTX1B STD 5 ng/ml**" was prepared using synthetic CTX1B chemically synthesized by M. Hirama et al. The concentration of CTX1B in the "**CTX1B STD 5 ng/ml**" standard was calibrated using a standard curve based on ELISA measurements of natural CTX1B performed and quantified by T. Kato and T. Yasumoto. Standard curves of natural and synthetic CTX1B are provided at the end of this manual.

* Please read this operation manual thoroughly before use and check that the attached reagents match the above accessory list.

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Abbreviations used in this manual.

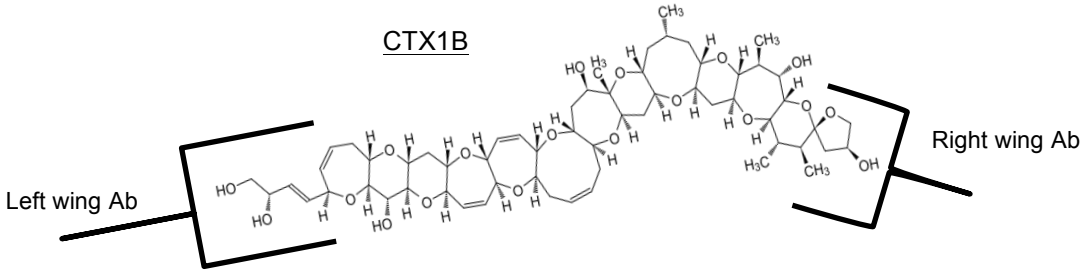
Alkaline phosphatase: ALP, Ciguatoxin: CTX, Enzyme-linked immunosorbent assay: ELISA

1. INTRODUCTION

In areas surrounded by subtropical and tropical coral reefs, more than 50,000 people in a year suffer from Ciguatera food poisoning (CFP). This CFP had been reported from Great Navigation Age of the 16th from the 17th century, but the cause of it was unknown until late years. But the causative organism, a species of “Dinoflagellate Gambierdiscus Toxicus”, was elucidated by Dr. Yasumoto at 1977 year. Afterwards, various studies of the ciguatera seafood poisoning, so called Ciguatoxins (CTXs), were performed by many researchers, and the result more than 20 congeners toxins were discovered all over the world.

Recently, Dr. Hirama and coworkers of Tohoku University in Japan chemically synthesized four congeners of CTX (CTX3C, CTX1B, 51-hydroxy-CTX3C, and 54-deoxy-CTX1B), which are mainly present in the Pacific Ocean. Subsequently, using synthetic hapten-KLH conjugates as antigens, Dr. Tsumuraya produced several kinds of monoclonal antibodies (mAb) that strongly bind to the right side and the left side of CTX molecules, and they successfully developed a highly sensitive immunochemical detection method (SANDWICH ELISA).

Based on these researches, a new ELISA kit “CTX-ELISA™1B” that detectable CTX1B and 54-deoxyCTX1B at the concentration range from 0.2 to 0.0005 ppb was deveroped, and commercialized as a first product in the world. Since the detection sensitivity is much superior to the FDA guidance level of 0.01 ppb, these kits will be useful not only for prevention of CFP but also for the epidemiological and physiological studies.



2. PRINCIPLE OF THE ASSAY

The "CTX-ELISA™1B" kit (Code number: 8100) is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of ciguatoxin1B (CTX1B) and 54-deoxyCTX1B in fish flesh, serum, plasma, etc. This kit contains sufficient reagents for 2 x 96 determinations.

Operation flow

1. Prepare **washing buffer**, **test sample** and **CTX1B standard solution** as directed.

⇓

2. Add 100 µL of **test sample**, **CTX1B standard solution,** and **D-1** to each well.
Incubate for 30 min at 37˚C.

⇓

3. Aspirate and wash 3 times with 200 µL/well of washing buffer.

⇓

4. Make **anti-CTX1B-ALP solution** as directed.

⇓

5. Add 100 µL of **anti-CTX1B-ALP solution** to each well and Incubate for 30 min at 37˚C.

⇓

6. Aspirate and wash 3 times with 200 µL/well of washing buffer.

⇓

7. Add 100~200 µL of **R-1** or a commercially available ALP fluorescent substrate solution to each well and incubate for 30 to 45 min at 37°C.

⇓

8. Measure A_{405} and A_{490} (or FI using a fluorescence micro-plate reader).

3. PRECAUTIONS

- **RESEARCH USE ONLY, NOT FOR FOOD INSPECTION AND CLINICAL DIAGNOSTIC USE.**
- Store the kit in the refrigerator (2~8˚C) promptly upon receipt and do not store in freezer.
- This kit should not be used past the expiration date indicated on the box**.**
- Detection kit is restricted only to the investigation of CTX1B and 54-deoxyCTX1B and can't detect CTX3C congeners.
- Do not expose reagents to excessive heat or light during storage and incubation.
- The reagents **W-1, D-1** and **D-2** contain surfactant, therefore strong stirring will generate persistent foam which is interfere with measurement, please stir slowly and gently.
- Reagents may contain antibiotics and antiseptics. Wear gloves while running the assay to avoid contact with sample and reagents. Please follow the appropriate disposal procedure established in each country or region.

4. STORAGE CONDITIONS

* Storage conditions for each part after opening are as follows, please observe this condition

Parts	Store condition
ELISA Assay Plate CTX-1B	Refrigerator (2~8˚C)
Anti-CTX1B-ALP, 250 µl/vial	Refrigerator (2~8˚C)
*1 CTX1B STD 5ng/mL, 100 µl/vial	Refrigerator (2~8˚C)
Plate Wash Buffer x20 Conc. **W-1**	Room temp.
STD/Sample diluent, **D-1**	Room temp.
Anti-CTX1B-ALP diluent, **D-2**	Room temp.
ALP Substrate Solution, **R-1** (pNPP)	Refrigerator (2~8˚C)
Adhesive plastic sheet	Room temp.

5. OTHER SUPPLIES REQUIRED

- Micro-plate reader capable of measuring absorbance (A) at 405 nm and 490 nm. If a 490nm filter is not available, measurement at only A_{405} may be useful.
- Multichannel 6 or 12 channel dispenser with disposable plastic tips to deliver 100-200 µL.
- Single channel precision pipettes with disposable tip to dispense 10-1,000 µL.
- Glass test tubes or vials for preparing CTX1B standard solutions and test sample solutions.
- Glass or plastic 1 litter bottle for preparing washing buffer.
- Disposable reagent reservoir tray to dispense washing buffer, Anti-CTX1B-ALP solution, and **R-1** with Multichannel Dispenser
- Ultrapure water.
- Acetone, Chemical grade, for extract CTX1B from fish flesh.
- Dimethyl sulfoxide (DMSO), chemical grade, for preparing the sample solution.
- Automated microplate washer, if available.
- Linear or logarithmic graph paper, or a computer with statistical analysis software for ELISA data.
- The following additional reagents and equipment are required for more sensitive assays. "AttoPhos®AP Fluorescence Substrate System; Cat. #S1000, Promega, Fitchburg, WI, USA" or "QuantiFluo™ Alkaline Phosphatase Assay Kit; Cat. #QFAP-100, Bioassay Systems, Hayward, CA, USA" as fluorescent substrate, and Fluorescent Micro-plate Reader.

6. REAGENTS PREPARATION

*Bring all reagents to room temperature before use.

① Washing buffer

Transfer the entire contents of **W-1** (50 mL) to the 1 L bottle. Dilute **W-1** to a final volume of 1 L with ultrapure water and mix thoroughly.

*The diluted washing buffer solution must be kept at room temperature before use.

*Do not use the washing buffer if it becomes turbid or precipitates are observed during storage.

② Anti-CTX1B-ALP solution

*Do not prepare more diluted "anti-CTX1B-ALP" solution than necessary.

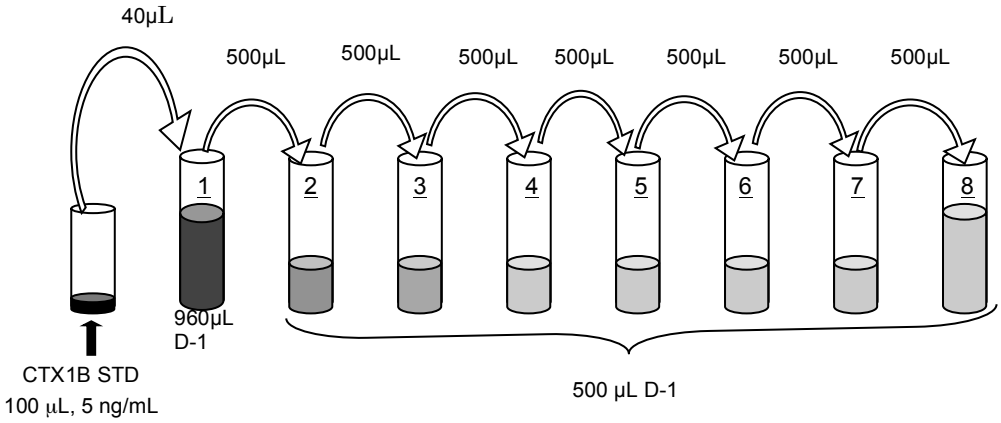
*Use 15 mL plastic test tube to prepare the solution.

- Dilute "**anti-CTX1B-ALP**" with **D-2** as indicated below just before use and use the solution as quickly as possible. The remaining "**anti-CTX1B-ALP"** should be stored in a refrigerator.
- **Colorimetric assay.** Dilute 100 fold
- **Fluorometric assay.** Dilute 200~400 fold

③ CTX1B standard solution

Pipette 960 µL of **D-1** into one glass test tube or vial and 500 µL of **D-1** into 7 glass test tubes or glass vials. Remove 40 µL of "**CTX1B STD 5 ng/ml**" and add to the first tube containing 960 mL of **D-1** to make a 200 pg/mL solution of CTX1B STD. Transfer half (500 µL) of this solution into the second tube and mix well before the next transfer. Next, 200~1.5 pg/mL standard solutions are prepared by serial 2-fold dilutions. These diluted solutions should be used within 30 minutes of preparation. The remaining **D-1** can be used as the zero standard (0 pg/mL).

The rest of "**CTX1B STD 5 ng/ml**" should be kept in refrigerator.



Vial No.	1	2	3	4	5	6	7	8
Conc. pg/mL	200	100	50	25	12.5	6.25	3.12	1.56

7. PREPARATION OF TEST SAMPLE

7-1. Fish flesh extracts

*This is a proposed method and users may develop a better extraction procedure.

① Transfer 1 g of fish flesh into a glass test tube, add 5 mL of acetone, then mince finely using an ultra-sonic vibrator, glass homogenizer, etc.

② Centrifuge at 20,000xg for 10 min at 4˚C, collect the supernatant using a pipette, transfer to an evaporation glass tube, then remove the acetone and water by evaporation in a vacuum or by air-blow drying.

③ Add 1 mL of DMSO to the residue and dissolve by stirring.

④ Centrifuge and collect the supernatant into a glass test tube or vial and store in a refrigerator as a test sample until use.

⑤ This DMSO extract should be diluted more than 20-fold with **D-1** just before the assay.

7-2. Blood preparation

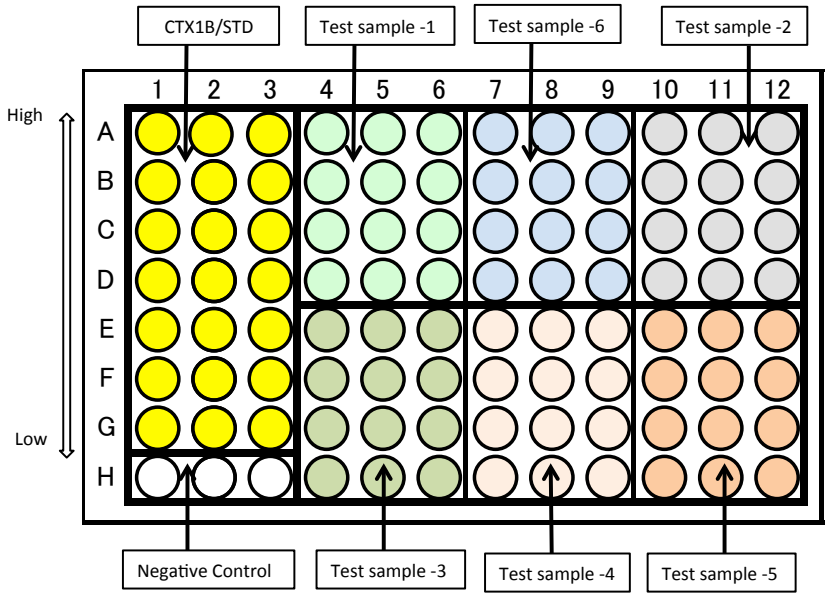
* Wear protective gloves, mask and eyeglasses when handling human blood.

① Collect 2 to 5 mL of blood without anti-coagulant from a patient or a fish, place in a glass centrifuge tube, and let it stand overnight in a refrigerator.

② Centrifuge at 1,500xg for 10 minutes at room temperature, transfer the whole supernatant (serum fraction) into a new glass tube, and use it as a test sample.

8. ASSAY PROCEDURE

* Warm all reagents at room temperature just before use.
* It is recommended that 3 wells be used to measure each test sample, standard solution and zero standard.
* Be careful not to disconnect the strip-wells from the microplate frame when removing the adhesive sheet and discard the liquid in the well.



8-1. Colorimetric assay

① Prepare **Washing Buffer**, **CTX1B standard solution** and **Test samples** as described section 6 and 7.

② Take out the ELISA Assay Plate from the aluminum pouch, and remove excess strip-wells from the micro-plate frame, then return them into the aluminum pouch and reseal it, and store in the refrigerator.

③ Add 100 µL of **CTX1B standard solution**, test sample and **D-1** as zero standard, in each defined well. It is recommended to use 3 wells for each concentration of standard solution and test sample. See the figure below.

④ Cover with the adhesive plastic sheet provided, and incubate for 30 minutes at room temperature or in 37˚C incubator.

⑤ Remove the adhesive plastic sheet carefully, and discard the buffer in all wells by aspirating or decanting.

⑥ Plate washing:

a. Add 200 µL of washing buffer in each well using multichannel dispenser.

b. Wash all wells by shaking with plate mixer.

c. Discard the buffer in all wells by aspirating or decanting.

d. Turn over the plate on a clean paper towel and tap the plate to blot the remaining buffer.

e. Repeat the above procedures three times.

⑦ Add 100 µL of diluted **Anti-CTX1B-ALP** solution into each well using multichannel dispenser, cover with adhesive plastic sheet, and incubate for 30 minutes at room temperature or 37˚C.

⑧ Repeat the above washing procedure ⑥

⑨ Add 100-200 µL of **R-1** in each well using multichannel dispenser, and incubate for 30 to 45 minutes at 37˚C. * If air bubbles remain in the wells, erase the bubbles by air blowing or by plate centrifuge, before measurement.

⑩ Measure the absorbance (A) of each well at λ 405 nm and 490 nm using a micro-plate reader. Subtract A_{490} from A_{405} to correct for optical imperfections in the micro-plate. Only A_{405} is also useful, if A_{490} is not available.

8-2. Highly sensitive fluorometric assay

① Follow the above procedures ①～⑧.

② Then, add 100 µL of ALP substrate solution written below, and measure the fluorescence intensity (FI).

* Usable fluorescent reagents:

"AttoPhos®AP Fluorescence Substrate System" Cat. #S1000, Promega;

"QuantiFluo™ Alkaline Phosphatase Assay Kit" Cat. #QFAP-100, Bioassay Systems.

③ All assay procedure should be performs according to technical bulletin of each substrate Kit.

9. CALCULATION OF RESULTS

Average the absorbance (A) (or the fluorescence intensities (FI)) of the standard solutions, from which subtract the averaged A (or FI) of the zero standard. Plot the corrected A (or FI) on the Y-axis against the concentration (pg/mL) of CTX1B on the X-axis of the graph paper, and draw the best-fit straight line as the standard curve. The concentration of the sample (CTX1B) can be determined from the X-axis by interpolation of A (or FI) on the Y-axis. If the sample was diluted, multiply the obtained interpolated value by the dilution factor to calculate amount of CTX1B in the sample.

If the A (or FI) values of a sample are higher than the highest value of the standard, try again after appropriately diluted with **D-1**.

The optimal standard curve can be obtained by statistical analysis using computer and the software for ELISA to give the concentration of the sample automatically.

10. TECHNICAL HINTS

- Glass vessels should be used in all processes. Do not use plastic test tubes or vials to prepare and to store the CTX1B Standard solution and the test sample. Disposable plastic tip is usable only for short time.
- For dilution of **Anti-CTX1B-ALP**, please use plastic test tube.
- When take out **R-1** solution from the bottle, pour directly into test tube or measuring cylinder without using pipette. It can not be used when the color of the solution shows a strong yellow.
- When adding standard solutions or test samples to the ELISA assay plate, please add in order from the lower concentration to the higher concentration.
- To avoid cross-contamination, pipettes and dispensing pipette tips should be carefully replaced. Separate reservoirs be used for each reagent.
- **CTX1B Standard solution** and **test samples** must be transferred to the plate well within 30

minutes after preparation.

- Use a new adhesive plate cover for every incubation step.
- If the test sample solution contains organic solvents more than 10%, the antigen-antibody reaction is suppressed. Therefore, their concentration should be reduced below 5% by dilution with **D-1**.
- If the test sample solution becomes turbid possibly due to contamination of lipid when diluted with **D-1**, the lipid should be removed by high-speed centrifugation since it might disturb the antigen-antibody reaction.

11. REFERENCE

Ciguatera and its off-shoots-Chance encounters en route to a molecular structure. Scheuer, P. J. *Tetrahedron*, **50**, 3 (1994).

Structures and configurations of ciguatoxin from moray eel *Gymnothorax javanicus* and its likely precursor from the dinoflagellate Gambierdiscus toxicus. Murata, M., Legrand, A. M., Ishibashi, Y., Fukui, M., Yasumoto, T.
J. Am. Chem. Soc. **112**, 4380 (1990).

Marine toxins. Yasumoto, T., Murata, M. *Chem. Rev*. **93**, 1897 (1993).

Structural elucidation of ciguatoxin congeners by fast-atom bombardment tandem mass spectroscopy. Yasumoto, T., Igarashi, T., Legrand, A.-M., Cruchet, P., Chinain, M., Fujita, T., Naoki, H.
J. Am. Chem. Soc. **122**, 4988 (2000).

The chemistry and biological function of natural marine toxins. Yasumoto, T.
Chem. Rec. **1**, 228 (2001).

Total synthesis of ciguatoxin CTX3C. Hirama, M., Oishi, T., Uehara, H., Inoue, M., Maruyama, M., Oguri, H., Satake, M.
Science, **294**, 1904 (2001).

Synthesis-based approach toward direct sandwich immunoassay for ciguatoxin CTX3C. Oguri, H., Hirama, M., Tsumuraya, T., Fujii, I., Maruyama, M., Uehara, H., Nagumo, Y.
J. Am. Chem. Soc. **125**, 7608 (2003).

Total synthesis of ciguatoxin and 51-hydroxyCTX3C. Inoue, M., Miyazaki, K., Ishihara, Y., Tatami, A., Ohnuma, Y., Kawada, Y., Komano, K., Yamashita, S., Lee, N., Hirama, M.
J. Am. Chem. Soc. **128**, 9352 (2006).

Production of monoclonal antibodies for sandwich immunoassay detection of Pacific ciguatoxins. Tsumuraya, T., Fujii, I., Hirama, M.
Toxicon, **56**, 797 (2010).

Ciguatera incidence and fish toxicity in Okinawa, Japan. Oshiro, N., Yogi, K., Asato, S., Sasaki, T., Tamanaha, K., Hirama, M., Yasumoto, T., Inafuku, Y.
Toxicon, **56**, 656 (2010).

First toxin profile of ciguateric fish in Madeira Arquipelago (Europe). Otero, P., Perez, S., Alfonso, A., Vale, C., Rodriguez, P., Gouveia, N. N., Gouveia, N., Delgado, J., Vale, P., Hirama, M., Ishihara, Y., Molgo, J., Botana, L. M.
Anal. Chem. **82**, 6032 (2010).

Detailed LC-MS/MS analysis of ciguatoxins revealing distinct regional and species characteristics in fish and causative alga from the Pacific. Yogi, K., Oshiro, N., Inafuku, Y., Hirama, M., Yasumoto, T.
Anal. Chem. **83**, 8886 (2011).

Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: Thiol strategy and detection using a sandwich ELISA. Tsumuraya, T., Takeuchi, K., Yamashita, S., Fujii, I., Hirama, M.
Toxicon, **60**, 348 (2012).

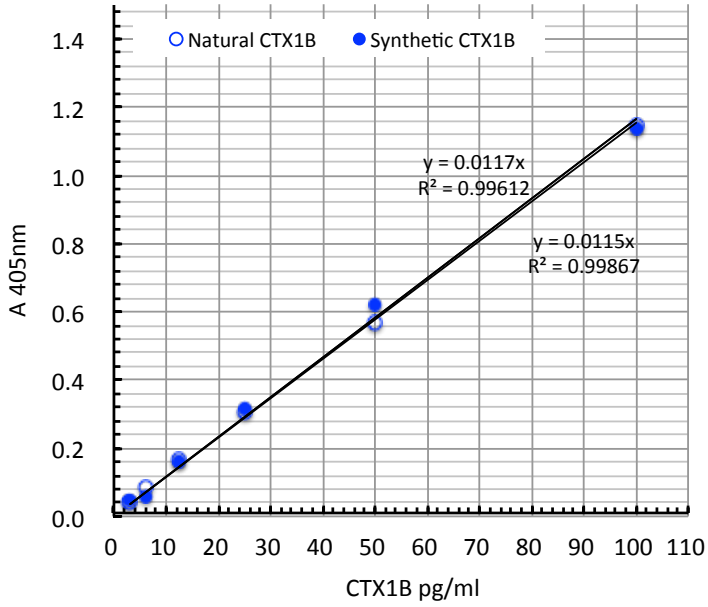
Preparation of anti-ciguatoxin monoclonal antibodies using synthetic haptens: Sandwich ELISA detection of ciguatoxins. Tsumuraya, T.,Fujii, I., Hirama, M.
J. ACAC. **97**, 373 (2014).

Practical route to the left wing of CTX1B and total syntheses of CTX1B and 54-deoxyCTX1B. Yamashita, S., Takeuchi, K., Koyama, T., Inoue, M., Hayashi, Y., Hirama, M.
Chem. Eur. J. **21**, 2621 (2015).

Highly Sensitive and Practical Fluorescent Sandwich ELISA for Ciguatoxins. Tsumuraya, T., Sato, T., Hirama, M., Fujii, I.,
Anal. Chem. **90**, 7318 (2018).

Manufacture: Cell Science Inc. (CSI); 82-16 Imozawa-Gongenmoriyama, Aoba-ku, sendai, 989-3212, JAPAN
Supplier: FUJIFILM Wako Pure Chemical Co.; 1-2 Doshomachi 3-Chome, Chuo-ku, Osaka 540-6605, JAPAN
www.cellscience.co.jp

12. COMPARISION BETWEEN THE CALIBRATION CURVES OF NATURAL AND SYNTHETIC CTX1B

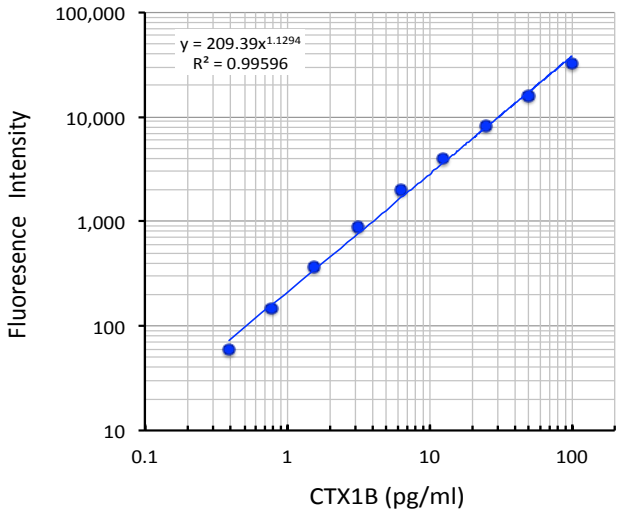


There are no difference in reactivity between the natural and the synthetic CTX1B.

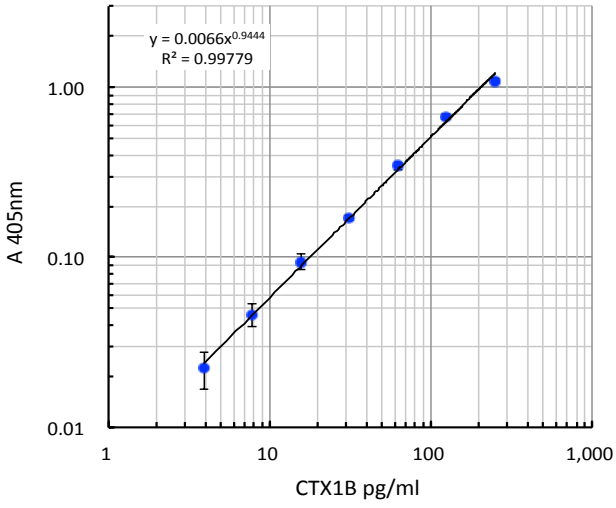
Caution: These data are provided for demonstration only. A standard curve should be generated for each assay.

13. TYPICAL STANDARD CURVES FOR COLORIMETRIC AND FLUOROMETRIC ASSAY

Fluorometric assay by "AttoPhos®AP Fluorescence Substrate System"



Colorimetric assay by R-1 (pNPP)



1. Detection limit of Colorietric assay and Fluorometric procedures are about 5pg/ml (0.005 ppb) and 0.5pg/ml (0.0005 ppb), respectively.
2. The Fluorometric assay was 10 times higher than the Colorimetric method, and its performance greatly exceeded the FDA guidance of 0.01ppb.

14. PERFORMANCE CHARACTERISTICS

Sensitivity: <3pg/ml
Detection limits were determined by repeated measurements of negative control (0 pg) and standards.

Assay Range: 5 to 200 pg / mL by Colorimetric assay, 0.5 to 100 pg / mL by Fluorometric assay.

Specificity: This ELISA kit can detect ciguatoxin1B and 54-deoxyCTX1B, but not cross-react with other ciguatoxins.

Reproducibility: CV value <15%
The coefficient of variation (CV%) of the measured value when repeatedly measuring standard antigens of three concentrations.

Calibration of "CTX1B STD 5 ng/ml": Concentration of CTX1B in "CTX1B STD 5 ng/ml" preparation was calibrated using natural CTX1B whose concentration was determined by T. Kato and T. Yasumoto et al. as a standard.