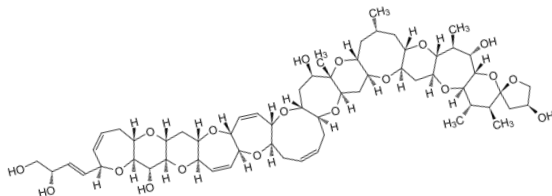


Ciguatoxin1B ELISA Kit



“CTX-ELISA™1B” Operation Manual Ver. 2

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 (Freeze dry powder)	1 tube
8117	CTX-1B STD 5 ng/mL ^{*1}	1 tube
--	Adhesive plastic sheet	6 sheets
--	Operation manual	1

Storage: Keep in the refrigerator (2~8°C) promptly upon receipt, freezing prohibit.

^{*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 standard was calibrated using a standard curve based on ELISA measurements of natural CTX1B that was 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 and check the attached reagents match the above accessory list before use.

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Abbreviations used in this manual.

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

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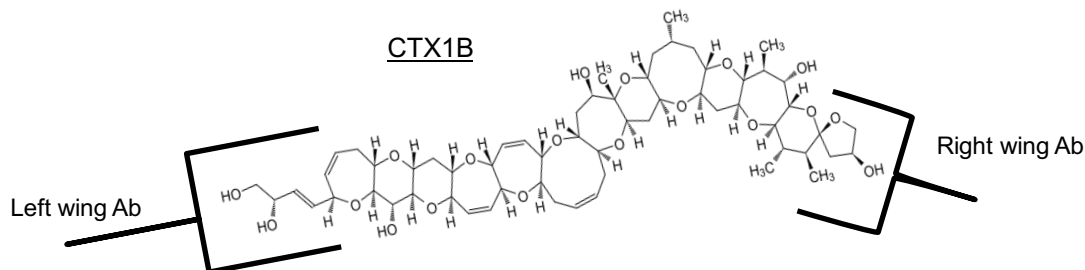
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1. INTRODUCTION

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. Hiram 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 quantitative range from 0.2 to 0.0003 ppb was developed, 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 μ L of **test sample**, **CTX1B standard solution**, and **D-1** to each well.
Incubate for 30 min at 37°C.
- ↓

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3. Aspirate and discard the liquid in each well
 ↓
 4. Add 100 μ L of **anti-CTX1B-ALP solution** to each well and Incubate for 30 min at 37°C.
 ↓
 5. Aspirate and wash 3 times with 200 μ L/well of washing buffer.
 ↓
 6. Add 200 μ L of **R-1** or a commercially available ALP fluorescent substrate solution to each well and incubate for 30 to 45 min at 37°C.
 ↓
 7. 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.**
- Expiration date is shown side on the box. If don't use immediately, store in refrigerator promptly upon receipt, freezing prohibit.
- Detection kit is restricted only to the investigation of CTX1B and 54-deoxy CTX1B, can't use for detection 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.

Reagents/Parts	Store condition
ELISA Assay Plate CTX-1B	Cool (2~8°C)
Anti-CTX1B-ALP, (Freeze dry powder)	Cool (2~8°C)
*1 CTX1B STD 5ng/mL, 100 μ L/vial	Cool (2~8°C)
Plate Wash Buffer x20 Conc. W-1	Cool or Room temp.
STD/Sample diluent, D-1	Cool or Room temp.
Anti-CTX1B-ALP diluent, D-2	Cool or Room temp.
ALP Substrate Solution, R-1 (pNPP)	Cool (2~8°C)
Adhesive plastic sheet	Room temp.

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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 μ L.
- Single channel precision pipettes with disposable tip to dispense 10-1,000 μ L.
- Glass test tubes or vials for preparing CTX1B standard solutions and test sample solutions.
- Glass or plastic 1 liter 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

*All reagents keep 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 keep at room temperature.

② Anti-CTX1B-ALP solution

- Dissolve the powder of “**anti-CTX1B-ALP**” with 250 μ L of **pure water**.
- The solution dilute with **D-2** as indicated below, and use as soon as possible.
Colorimetric assay. 100 folds dilution; **Fluorometric assay.** 200~400 folds dilution
- The remaining solution should be saved in a refrigerator, and use it up within 2 months.

*Do not prepare excess diluent more than necessary. Recommend plastic test tube to preparation.

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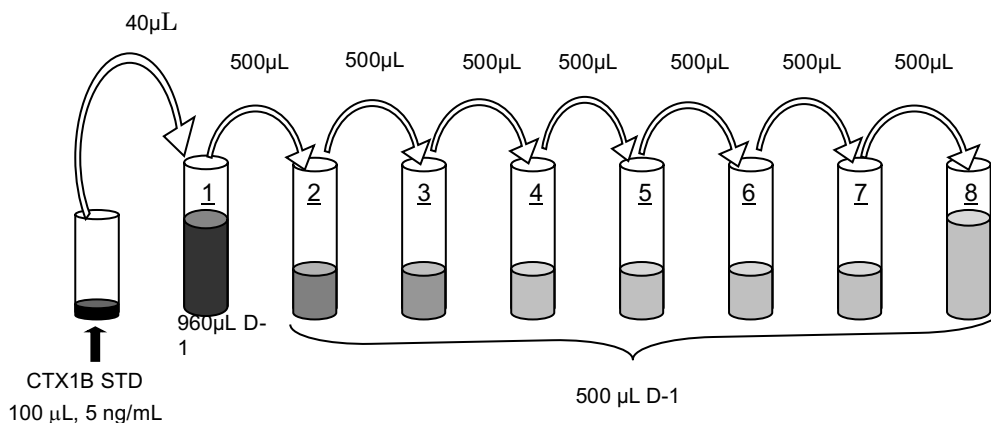
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③ CTX1B standard solution

Pipette 960 μL of **D-1** into one glass test tube or vial, and dispense 500 μL of **D-1** into 7 each glass test tube or glass vial. And add 40 μL of "CTX1B STD 5 ng/ml" to the first tube containing 960 μL of D-1 to make a 200 pg/mL of CTX1B standard solution. Transfer half (500 μL) of this solution into the second tube and mix well before the next transfer. Next, 200~1.56 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 "**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 ultrasonic vibrator, glass homogenizer, etc.
- ② Centrifuge at 20,000 \times g 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 vacuum evaporation 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.

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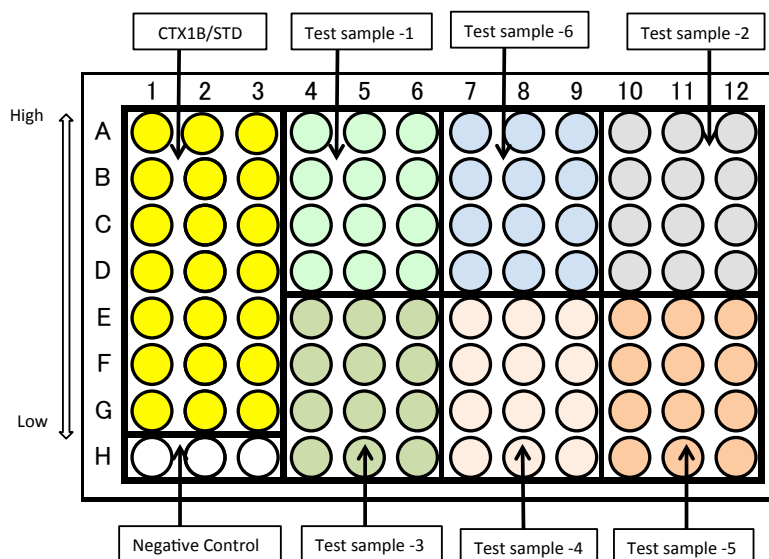
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- ① Collect 2 to 5 mL of blood without anti-coagulant from a patient or a fish, place in a glass centrifuge tube, and let it stand overnight in a refrigerator.
- ② Centrifuge at 1,500xg for 10 minutes at room temperature, transfer the whole supernatant (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** and **Test samples** as described section 6 and 7.
- ② Take out the ELISA Assay Plate from 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 μ L of **CTX1B standard**, **test sample** and **D-1** as negative control, in each defined well. It is recommended to use 3 wells for each concentration of standard and test sample. See the figure above.
- 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 by aspirating or decanting.
- ⑥ Add 100 μ L of diluted **Anti-CTX1B-ALP** solution into each well using multichannel dispenser, cover

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with adhesive plastic sheet, and incubate for 30 minutes at room temperature or 37°C.

- ⑦ Remove the adhesive plastic sheet carefully, and discard the buffer by aspirating or decanting.
- ⑧ Wash the plate 3 times by repeating below procedure. This washing operation can be replaced by an automated microplate washer device.
 - a. Add 200 μ L 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. Three times repeat the above procedures.
- ⑨ Add 200 μ L 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 Add 100 μ L 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 perform 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.
- When dilution of **Anti-CTX1B-ALP**, please use plastic test tube.
- Pour the R-1 solution directly from the bottle into the measuring container without using a pipette. Yellow colored solutions cannot be used.
- When adding standard solutions or test samples to the ELISA plate, please add in order from the lower 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.

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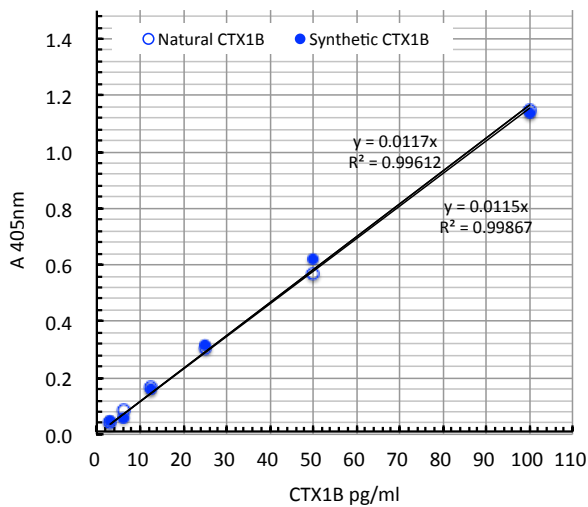
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12. COMPARISON BETWEEN THE CALIBRATION CURVES OF NATURAL AND SYNTHETIC CTX1B

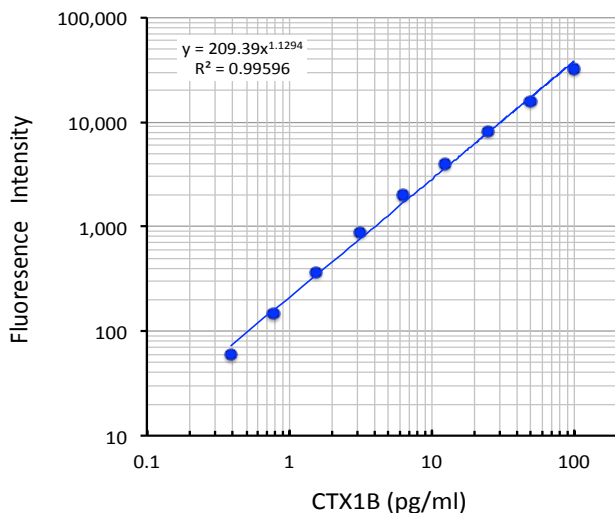


There are no difference in reactivity between the natural and the synthetic CTX1B.

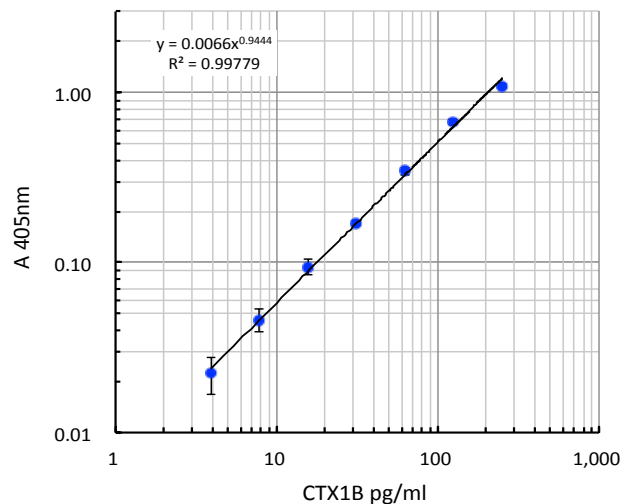
Caution: These data are provided for demonstration only. A standard curve should be generated for each assay.

13. TYPICAL STANDARD CURVES FOR COLORIMETRIC AND FLUOROMETRIC ASSAY

Fluorometric assay by "AttoPhos® AP Fluorescence Substrate System"



Colorimetric assay by R-1 (pNPP)



1. Quantitative limit of Colorimetric assay and Fluorometric procedures are about 3 pg/ml (0.003 ppb) and 0.3 pg/ml (0.0003 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.

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14. PERFORMANCE CHARACTERISTICS

Detection limit: <1 pg/ml by colorimetric assay.

Detection limits were determined by repeated measurements of negative control (0 pg) and standards.

Assay Range: 3 to 100 pg /mL by Colorimetric assay, 0.3 to 200 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.

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"AttoPhos® AP" is a registered trademark of Promega, USA.

"QuantiFluo™" is a trademark of Bioassay Systems, USA.

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