ELISA kit for measuring crustacean protein in food products

Crustacean kit Ⅱ “Maruha Nichiro“

- instruction manual -

For Laboratory Use Only.

Storage Conditions: Store between 2-8℃

Principle of the Assay

The **Crustacean kit Ⅱ "Maruha Nichiro"**, designed to measure the concentration of crustacean protein in food products employs the quantitative sandwich enzyme immunosorbent assay technique. A monoclonal antibody specific for black tiger tropomyosin (BTTM) has been pre-coated onto a microplate. Standards and samples are pipetted into the wells. After any unbound substances have been washed away, an HRP-conjugated polyclonal antibody specific for BTTM is added to the wells. Following a wash to remove any unbound HRP- conjugated antibody, the remaining conjugate is allowed to react with the substrate 3, 3', 5, 5' –tetramethylbenzidine. The reaction is stopped by the addition of acidic solution, and the absorbance of the resulting yellow products is then measured at 450/600-650 nm. The absorbance is proportional to the concentration of crustacean protein. A standard curve is constructed by plotting absorbance values versus crustacean protein concentrations of calibrators, and the concentrations of crustacean protein in the test samples are determined by using this standard curve.

Summary of the Procedure

Add 100 µL of diluted samples to the wells

⇩ incubate for 90 min at room temperature (20-25℃)

Wash the wells

⇩

Add 100 µL of HRP-conjugated anti-BTTM polyclonal antibody

⇩ incubate for 30 min at room temperature (20-25℃)

Wash the wells

⇩

Add 100 µL of Substrate Reagent

⇩ incubate for 20 min at room temperature (20-25℃)

Add 100 µL of Stop Solution

⇩

Measure absorbance at 450/600-650 nm

Materials Provided

Bring all reagents to room temperature before use.

A) Microtiter plate: One microtiter plate supplied ready to use, with 96 wells (12 strips of 8wells), shipped in a foil, zip-lock bag with a desiccant pack. Wells coated with anti-BTTM monoclonal antibody as the capture antibody.

B) Calibration Standard: One vial containing 1.8 mL of Crustacean protein in a buffer to provide a control value of 50 ng/mL.

C) HRP-conjugated Detection Antibody: One bottle containing 13 mL of HRP (horseradish peroxidase)-conjugated anti-BTTM polyclonal antibodies with preservative. Ready to use.

D) Substrate Reagent: One bottle containing 13 mL of stabilized 3, 3', 5, 5' -tetramethylbenzidine (TMB). Ready to use.

E) Stop Solution: One bottle containing 13 mL of 2 N sulfuric acid.
(CAUTION THIS SOLUTION IS ACIDIC) Avoid contact of this solution with eyes and skin. In case of skin contact, wash immediately with copious amounts of water. A mild soap should be used. In case of eye contact, flush generously for at least 15 minutes with water. Seek medical attention if the irritation persists or is severe.

F) Dilution Buffer 1: One bottle containing 100 mL of Dilution Buffer 1 with preservative. Ready to use.

G) Wash buffer concentrate solution (10X): One bottle containing 100 mL of concentrated wash buffer solution.

H) Extraction solution concentrate ① (10X): One bottle containing 100 mL of concentrated extraction solution A.

I) Extraction solution concentrate ② (10X): One bottle containing 100 mL of concentrated extraction solution B.

J) Extraction solution concentrate ③ (10X): One bottle containing 100 mL of concentrated extraction solution C.

Additional Materials Required

Blender: for sample preparation.

Graduated cylinder

Clean test tubes or small microtubes: for dilution of the calibration standard and samples.

Precision micropipettes: 20-200 µL and 200-1000 µL precision micropipettes with disposable tips.

Disposable paper towels

Distilled or deionized water

Vortex mixer

Microtiterplate reader: capable of measuring absorbance in 96-wells plates at dual wavelengths of 450/600-650 nm.

Precautions and Recommendations

Allow all components to come to room temperature before use.

Do not use kit components beyond the indicated kit expiration date.

Do not mix reagents from different kits.

Do not mouth pipette or ingest any of the reagents.

Dispose of tetramethylbenzidine (TMB)-containing solutions in compliance with local regulations.

Do not use solutions if they contain any precipitate or have become cloudy.
Exception: **I) Extraction solution concentrate ① (10X)** may precipitate during refrigerated storage but will dissolve upon warming. Any precipitates must be fully dissolved prior to diluting the concentrate to the final working strength.

Do not add azides to the samples or to any of the reagents. The calibration standard and some reagents already contain a preservative.

Treat all reagents and samples as potentially allergenic materials.

Do not use the same pipette to obtain from substrate and enzyme conjugate from their bottles, as this will contaminate these solutions. Always determine the required volumes of these reagents and dispense the volumes required accordingly into clean test tubes just prior to use.

Do not pour or return unused enzyme conjugate or substrate back into their bottles.

Ensure all glassware, plasticware, and storage bottles have been thoroughly cleaned to prevent any cross contamination from possible allergenic material or reagents from other test kits or previous test runs. This is especially the case when any extraction additives may have been used for specific assay extractions.

Storage Conditions

Reagents, microwell plate, and bottled components:

Store between 2-8 ℃. ***DO NOT FREEZE ANY OF THE KIT COMPONENTS.***

Valid for six month from the production date. The production date is printed on the outer case.

Avoid exposure of the kit and the components to direct sunlight at all times, as some reagents are light sensitive.

Reagent Preparation

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **G)Wash buffer concentrate solution (10X)**, **Extraction solution concentrate①－③ (10X)**, and Dilution Buffer 2.

Wash Buffer

Prepare a working solution of Wash Buffer by adding 100 mL of the **G)Wash buffer concentrate solution (10X)** to 900 mL of distilled (deionized) water. Mix well.

Extraction Solution

Prepare a working solution of Extraction Solution by mixing well the component concentrates [**H)Extraction solution concentrate ① (10X)**/ **I)Extraction solution concentrate ②(10X)**/ **J)Extraction solution concentrate③(10X)**/ distilled (deionized) water at a ratio of 2:2:2:14.

Preparation Example

H)Extraction solution concentrate① (10X)	30 mL
I)Extraction solution concentrate② (10X)	30 mL
J)Extraction solution concentrate③ (10X)	30 mL
distilled (deionized) water	210 mL
Total	300 mL

Dilution Buffer 2

Prepare a working solution of Dilution Buffer 2 by adding 1 mL of the Extraction solution to 19 mL of **F)Dilution Buffer 1** just before the assay. Mix well.

Calibration Standard

Use the **D)Calibration Standard** to produce a dilution series (see below). Mix the contents of each tube thoroughly before the next transfer. The 50 ng/mL standard (Std. 1) serves as the highest concentration of the standard. Dilution Buffer 2 serves as the zero standard (Blank).

Prepare the dilution series just before the assay.

Preparation Example

	Volume of Standard	Dilution Buffer 2	Concentration
Std. 1	400 µL of Calibration Standard	-	50.00 ng/mL
Std. 2	400 µL of Std. 1 (50.00 ng/mL)	400 µL	25.00 ng/mL
Std. 3	400 µL of Std. 2 (25.00 ng/mL)	400 µL	12.50 ng/mL
Std. 4	400 µL of Std. 3 (12.50 ng/mL)	400 µL	6.25 ng/mL
Std. 5	400 µL of Std. 4 (6.25 ng/mL)	400 µL	3.12 ng/mL
Std. 6	400 µL of Std. 5 (3.12 ng/mL)	400 µL	1.56 ng/mL
Std. 7	400 µL of Std. 6 (1.56 ng/mL)	400 µL	0.78 ng/mL
Blank	-	400 µL	0 ng/mL

Note: Change tips for every dilution when using a repeating pipette.

Sample preparation

The sample must be blended/ground to a fine consistency to provide a homogeneous mixture.

Weight out 1 gram of finely ground sample and add 19 mL of the Extraction solution. Place the sample on a shaker and then shake it for 12-17 hours at room temperature for extraction. After the extraction, centrifuge at 3000 x *g* for 20 min at room temperature. Check the pH of the sample extract and, if necessary, adjust it to the neutral range (pH 6.0-8.0) by using pH-indicator paper. Filter the supernatant through filter paper or by use of a similar filtration device. The sample is now ready to be tested by the kit.

Samples are 20-fold diluted with Dilution Buffer 1 (50 µL of sample + 950 µL of Dilution buffer 1), and the samples are then added to the wells immediately.

If samples generate values higher than the highest standard, further dilute the samples with Dilution Buffer 2 and repeat the assay.

Assay Procedure

Bring all reagents to room temperature before use.

1. Dilute Calibration Standards and Samples.

2. Remove the Microtiter plate from the foil pouch.

3. Pipette 100 µL of Calibration Standards (Std.1 - Std.7, Blank) and diluted samples, in triplicate, into the appropriate wells.

4. Incubate the plate at room temperature (20-25 ℃) for 90 minutes.

5. Wash 5 times by filling each well with Wash buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

6. Add 100 µL of HRP-conjugated Detection Antibody to each well.

7. Incubate the plate at room temperature (20-25 ℃) for 30 minutes.

8. Wash 5 times by filling each well with Wash buffer (350 mL) again by using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

9. Add 100 µL of Substrate Reagent to each well.

10. Incubate the plate at room temperature (20-25 ℃) for 20 minutes.

11. Add 100 µL of Stop solution to each well.

12. Measure the absorbance of each well by using a microplate reader at dual wavelengths of 450/620 nm. Wells must be read within 30 minutes of adding the Stop solution.

Note: Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Calculation

Average the triplicate readings for each standard and sample. Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation. To determine the crustacean protein concentration of each sample, first find the absorbance values on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line down to the x-axis and read the corresponding crustacean protein concentration. The concentration read from the standard curve must be multiplied by the dilution factor (400 or greater).

Most microtiter plate readers automatically perform calculations of analyte concentrations. The calibration curve is constructed by the plate reader, which plots the absorbance (Y) of calibrators versus the log of the known concentration (X) of calibrators by using the four-parameter function.

Literature Cited

1) Seiki, K., Oda, H., Yoshioka, H., Sakai, S., Urisu, A., Akiyama, H., Ohno, Y. A reliable and sensitive immunoassay for the determination of crustacean protein in processed foods. *J. Agric. Food Chem.* 2007, **55**, 9345-9350.