Test Instruction

Molluscs (Tropomyosin)
ELISA

96/48 Tests

Enzyme Immunoassay
for the Quantitative
Determination of
Mollusc Tropomyosin in Food

Cat.-No.: MOL-E01/E04

Version: February 21st, 2019

This document represents a combined test instruction for the products MOL-E01 (96 well) and MOL-E04 (48 well).

Immunolab GmbH

Otto-Hahn-Str. 16
D-34123 Kassel
Germany

Phone: +49 (0) 561 491742-0
Fax: +49 (0) 561 491742-20
E-Mail: info@immunolab.de

www.immunolab.de

Sensitivity	1.7 ppb
Recovery	74-110%
Incubation Time	60 min

General Information

Not only because of their cross-reactivity to house dust mites and crustaceans, molluscs represent an important group of food allergens. In this regard tropomyosin, which can be found in all common mollusc species, is the most important protein. In cooked and uncooked mollusc extracts this protein partly represents a high amount of total protein.
For mollusc allergic persons hidden mollusc proteins in food are a critical problem. Already very low amounts of the allergen can cause allergic reactions, which may lead to anaphylactic shock in severe cases. Because of this, mollusc allergic persons must strictly avoid the consumption of mollusc containing food. Cross-contamination, mostly in consequence of the production process, is often noticed. This explains why in many cases the existence of mollusc residues in food cannot be excluded. For this reason sensitive detection systems for mollusc residues in foodstuffs are required.
The **Immunolab Molluscs** (Tropomyosin) **ELISA** represents a highly sensitive detection system for tropomyosin (from helix aspersa) and is particularly capable of the quantification of mollusc residues in fish products, soups, dressings, bakery products and meat products.

Principle of the Test

The **Immunolab Molluscs** (Tropomyosin) quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against mollusc tropomyosin is bound on the surface of a microtiter plate. Tropomyosin containing samples or standards are given into the wells of the microtiter plate. After 20 minutes incubation at room temperature, the wells are washed with diluted washing solution to remove unbound material. A peroxidase conjugated second antibody directed against tropomyosin is given into the wells and after 20 minutes of incubation the plate is washed again. A substrate solution is added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development is inhibited by the addition of a stop solution, and the colour turns yellow. The yellow colour is measured photometrically at 450 nm. The concentration of tropomyosin is directly proportional to the colour intensity of the test sample.

Precautions

Full compliance of the following good laboratory practices (GLP) will determine the reliability of the results:

1. Prior to beginning the assay procedure, bring all reagents to room temperature (20-25°C).

2. All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.

3. Once the assay has been started, all subsequent steps should be completed without interruption and within the recommended time limits.

4. Replace caps in all the reagents immediately after use. Do not interchange vial stoppers.

5. Use a separate disposable tip for each specimen to prevent cross-contamination.

6. All specimens and standards should be run at the same time, so that all conditions of testing are the same.

7. Do not mix components from different batches.

8. Do not use reagents after expiration date.

9. Check both precision and accuracy of the laboratory equipment used during the procedure (micropipets, ELISA reader etc.).

Health and safety instructions

1. Do not smoke or eat or drink or pipet by mouth in the laboratory.

2. Wear disposable gloves whenever handling patient specimens.

3. Avoid contact of substrate and stop solution with skin and mucosa (possible irritation, burn or toxicity hazard). In case of contact, rinse the affected zone with plenty of water.

4. Handling and disposal of chemical products must be done according to good laboratory practices (GLP).

Reagents

The kit contains reagents for 96/48 determinations. They have to be stored at 2-8°C. Expiry data are printed on the labels of the bottles and the outer package.

1. Microtiter plate consisting of 12/6 strips with 8 breakable wells each, coated with anti-tropomyosin antibodies.

2. Tropomyosin Standards (0; 10; 40; 100; 400 ppb of tropomyosin): 5 vials with 2.0 mL each, dyed red, ready-to-use.

3. Conjugate (anti-tropomyosin-peroxidase): 15/7.5 mL, dyed red, ready-to-use.

4. Substrate Solution (TMB): 15 mL, ready-to-use.

5. Stop Solution (0.5 M H_2SO_4): 15 mL, ready-to-use.

6. Extraction and sample dilution buffer (Tris): 2/1 x 120 mL as 10x concentrate, dyed red. Dilute 1+9 with distilled water. Stored at 4°C the diluted buffer is stable for at least one week. If during the cold storage crystals precipitate, the concentrate should be warmed up to 37°C for 15 minutes.

7. Washing Solution (PBS + Tween 20): 60 mL as 10x concentrate. Dilute 1+9 with distilled water. Stored at 4°C the diluted buffer is stable for at least 4 weeks. If during the cold storage crystals precipitate, the concentrate should be warmed up to 37°C for 15 minutes.

8. Plastic bag to store unused microtiter strips.

9. Instruction Manual.

Additional Instrumentation and Reagents (not provided)

Instrumentation

- 100 - 1000 µL micropipets
- Volumetric flask
- Analytical balance
- Mortar, mixer
- Water bath
- Centrifuge
- ELISA reader (450 nm)

Reagents

- double distilled water

Sample Preparation

Due to high risk of cross-contamination all applied instruments like applicator, mortar, glass vials etc. have to be **cleaned thoroughly** before and after each sample. Tropomyosin could adhere to different surfaces. To identify possible cross-contamination caused by previous extractions it is strongly recommended to note the sequence of the extractions.

The following sample preparation should be applied for solid samples:

1. To maximize homogeneity and representativeness of the sample drawing, a minimum of 5 g sample should be pulverized finely in a mortar, impact mill etc.
2. 1 g of the homogenized mixture is suspended in 20 mL of **pre-diluted** extraction and sample dilution buffer. Afterwards the suspension is incubated for 15 min in a preheated water bath at **40°C**. To ensure good homogeneity, the samples should be shaken every two minutes.
3. The samples are centrifuged for 10 minutes at 2000 g. If it is not possible to separate the supernatant from the precipitate completely, the suspension should be filtrated if necessary.
4. 100 µL of particle-free solution are applied per well. If the results of a sample are out of the measuring range, further dilution with the **pre-diluted** extraction and sample dilution buffer is necessary. The additional dilution has to be considered when calculating the concentration.

The following sample preparation should be applied for liquid samples:

1 mL of liquid sample is diluted in 19 mL of **pre-diluted** extraction and sample dilution buffer. Afterwards the suspension is incubated for 15 min in a preheated water bath at **40°C**. To ensure good homogeneity, the samples should be shaken every two minutes. The process is continued at point 3 of solid sample extraction process.

Procedure

The washing solution is supplied as 10x concentrate and has to be **diluted** 1+9 with double distilled water before use.

In any case the **ready-to-use** standards provided should be determined twofold. When samples in great quantities are determined, the standards should be pipetted once before the samples and once after the samples. For final interpretation the arithmetic mean is used for calculation.

In consideration of GLP and quality control requirements a duplicate measurement of samples is recommended.

The procedure is according to the following scheme:

1. Prepare samples as described above.
2. Pipet 100 µL **ready-to-use** standards or prepared samples in duplicate into the appropriate wells of the microtiter plate.
3. Incubate for 20 minutes at room temperature.
4. Wash the plate three times as follows: Discard the contents of the wells (dump or aspirate). Pipet 300 µL of diluted washing solution into each well. After the third repetition empty the wells again and remove residual liquid by striking the plate against a paper towel. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbencies.
5. Pipet 100 µL of conjugate (anti-tropomyosin-peroxidase) into each well.
6. Incubate for 20 minutes at room temperature.
7. Wash the plate as outlined in 4.
8. Pipet 100 µL of substrate solution into each well.
9. Allow the reaction to develop in the dark (e.g. cupboard or drawer; the chromogen is light-sensitive) for 20 minutes at room temperature.
10. Stop enzyme reaction by adding 100 µL of stop solution (0.5 M H_2SO_4) into each well. The blue colour will turn yellow upon addition.
11. After thorough mixing, measure absorbance at 450 nm (reference wavelength 620 nm), using an ELISA reader. The colour is stable for 30 minutes.

Calculation of results

The ready-to-use standards are prepared for a direct determination of sample concentrations. The dilution of samples in the extraction process as described in the above stated sample preparation procedure is already considered. Additional dilution due to high sample concentration has to be accounted for.

1. Calculate the average optical density (OD 450 nm) for each set of reference standards or samples.
2. Construct a standard curve by plotting the mean optical density obtained for each reference standard against its concentration in ppb on semi-log graph paper with the optical density on the vertical (y) axis and the concentration on the horizontal (x) axis. Alternatively the evaluation can be carried out by software. In this case the 4-parameter method should be preferred.
3. Using the mean optical density value for each sample, determine the corresponding concentra-

tion of tropomyosin in ppb from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

The determined amount of tropomyosin [ppb] can be used to calculate the amount of the corresponding mollusc raw product (**wet weight**). Therefore the amount of tropomyosin has to be multiplied with a conversion factor (F).

The following conversion factors were determined by validation experiments:

Grapewine snail (Helix aspersa), blanched	550
Grapewine snail (Helix pomatia), blanched	860
Abalone (Haliotis), raw	200
Abalone (Haliotis), cooked	580
Scallop (Pecten jacobaeus), raw	4850
Scallop (Pecten jacobaeus), cooked	6580
Green lipped mussel (Pema canalic.), raw	8620
Green lipped mussel (Pema canalic.), cooked	11230
Blue mussel (Mytilus edulis), raw	14990
Blue mussel (Mytilus edulis), cooked	16740
Carpet mussel (Venerupis pull.), raw	4965
Carpet mussel (Venerupis pull.), cooked	6190
Oyster (Ostreidae), raw	2940
Oyster (Ostreidae), cooked	4816
Octopus (Octopoda), raw	1360
Octopus (Octopoda), cooked	1360
Squid (Theutida), raw	700
Squid(Theutida), cooked	670

Typical Standard Values

The following table contains an example for a typical standard curve. The binding is calculated as percent of the absorption of the 400 ppb standard. These values are only an example and should not be used instead of the standard curve which has to be measured in each new test.

Tropomyosin (ppb)	% binding of 400 ppb
400	100
100	36
40	18
10	7
0	3

Performance

Sensitivity

The limit of detection (LOD) of the **Immunolab Molluscs** (Tropomyosin) **test** is 1.7 ppb (Tropomyosin, helix aspersa).

Validation experiments with common matrices resulted in the following LODs [ppb]:

Soy sauce	0.3
Vegetable soup	2.1
Bakery products	3.9
Fish	1.9
Meat	3.1

The limit of quantification (LOQ) of the Immunolab Tropomyosin test is 10 ppb.
Due to the variety of sample matrices and their influence on the blank, results less than the LOQ should be treated as negative.

Precision

Intra-assay Precision	5-6%
Inter-assay Precision	3%

Recovery

Mean recovery was determined by spiking samples with different amounts of tropomyosin:

Soy sauce	74%
Vegetable soup	88%
Bakery products	100%
Fish	103%
Meat	110%

Linearity

The serial dilution of spiked samples (soy sauce, vegetable soup, bakery products, fish, meat) resulted in dilution linearity of 89-107%.

Cross reactivity

For the following foods no cross-reactivty could be detected:

Apricot	Potato	Plum
Bean, white	Chervil	Pine seed
Buckwheat	Chickpea	Pistachio
Cashew	Cherry	Rice
Cayenne	Kiwi	Beef
Chili	Coconut	Bovine gelatin
Cumin	Cress	Rye
Cod	Pumpkin seed	Saccharose
Egg	Caraway	Ewe's milk
Pea	Lamb	Pork
Egg white powder	Lentil	Celery
Peanut	Lupin	Mustard
Fish gelatin	Corn	Sesame
Barley	Macadamia	Soy
Gliadin	Almond	Soy lecithin
Guar gum	Chestnut	Sunflower seed
Oats	Poppy seed	Tofu
Hazelnut	Nutmeg	Tomato
Isinglass	Bell pepper	Cow's milk
Chicken	Brazil nut	Walnut
Carob gum	Pecan nut	Wheat
Cocoa	Pepper	Goat's milk
Carrot	Peach	Onion