INSTRUCTION FOR USE

SENSI*Spec* ELISA Fish (Parvalbumin) 96/48 Tests

(Cat. nr. HU0030008 / HU0030032)

Enzyme Immunoassay for the Quantitative Determination of Fish in Food

Sensitivity (Cod)	1.4 ppm
Recovery	93-117%
Incubation Time	60 min

1. GENERAL INFORMATION

Fishes belong to the most frequent elicitors of food allergies. The allergies are predominantly induced by the low-molecular, calcium-binding muscle protein parvalbumin. The protein is characterized by its high heat resistance and stability against denaturing agents and proteolytic enzymes. Predominantly in regions with a high consumption of fish like Scandinavia, Japan or the Mediterranean countries, fish allergies represent a heavy health problem. The symptoms are ranging from inflammation of the skin over gastrointestinal and respiratory problems up to live-threatening anaphylactic shock. In spite of the high biodiversity most patients react with allergic symptoms to several fish species due to the high cross-reactivity between the fish allergens.

For fish-allergic persons hidden fish allergens in food are a critical problem. Already very low amounts of fish can cause allergic reactions, which may lead to anaphylactic shock in severe cases. Because of this, fish-allergic persons must strictly avoid the consumption of fish containing food. Cross-conta-mination, mostly in consequence of the production process, is often noticed. This explains why in many cases the existence of fish residues in food cannot be excluded. For this reason sensitive detection systems for fish residues in food-stuffs are required.

The **SENSI*Spec* ELISA Fish (Parvalbumin)** represents a highly sensitive detection system for fish, based on the trans-species allergen parvalbumin. It is particularly capable of the quantification of fish residues in wine, soups, sauces, crackers, surimi and asia products.

2. PRINCIPLE OF THE TEST

The **SENSI*Spec* ELISA Fish (Parvalbumin)** is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against fish proteins is bound on the surface of a microtiter plate. Fish 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 un-bound material. A peroxidase conjugated second antibody directed against fish proteins 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 terminated by the addition of a stop solution, and the colour turns yellow. The yellow colour is measured photometrically at 450 nm. The concentration of fish is di-rectly proportional to the colour intensity of the test sample.

3. 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.).

4. 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).

5. 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-fish antibodies.
2) Cod Standards (0; 4; 10; 40; 100 ppm of cod): 5 vials with 2.0 mL each, dyed red, ready-to-use.
3) Conjugate (anti-fish-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.

6. ADDITIONAL INSTRUMENTATION AND REAGENTS (NOT PROVIDED)

6.1 Instrumentation

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

6.2 Reagents

- double distilled water

7. 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. Fish proteins could adsorb 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 60°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 60°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.

Attention:

- *Do not shake the final extract to prevent from re-suspension.*
- *If after centrifugation a third layer at the top appears due to a high fatty matrix, only the middle aqueous phase should be applied to the wells.*

8. 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-fish-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.

9. 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 cod reference standard against its concentration in ppm on semi-log graph paper with the optical density on the vertical (y) axis and the cod 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 equivalent concentration of cod in ppm from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.
4) If the fish species of the sample is known, the amount of the appropriate species can be calculated by multiplying the test result (cod) with a species specific conversion factor:

Fish species	Conversion factor (F)
Bass	5.0
Carp	2.6
Catfish	1.7
Coalfish	3.0
Devilfish	274
Eel	29
Flounder	7.1
Haddock	21
Hake	12
Herring (smoked)	13
Mackerel (smoked)	50
Perch	5.2
Pike	0.3
Plaice	2.4
Red mullet	7.5
Redfish	103
Red Snapper	29
Salmon	1.7
Samlet	1.7
Sardine	101
Shark catfish	4.2
Spined loach	32
Swordfish	1250
Trout	2.0
Tuna	370
Turbot	27
Zander	11

It has to be considered that the standardisation as well as the conversion factors relate to fresh fish. For the interpretation of the test results, the grade of process of the respective food sample has to be accounted for. Validation experiments showed that cooked cod meat (20 min) resulted in a reactivity of 25% compared to fresh cod.

Please note: *for calculation of results, it is suggested to use the Excel spreadsheet that is available in the Document Repository section of the Gold Standard Diagnostics web site (www.goldstandarddiagnostics.com).*

10. 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 100 ppm 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.

Cod (ppm)	% binding of 100 ppm
100	100
40	63
10	24
4	14
0	7

11. PERFORMANCE

11.1 Sensitivity

The limit of detection (LOD) of the **SENSI*Spec* ELISA Fish (Parvalbumin) test** is 1.4 ppm (cod) for the standard curve.

Validation experiments with common matrices resulted in the following LODs [ppm].

Wine (red)	1.5
Soup	1.3
Worcester Sauce	0.3
Asia Sauce	2.1
Cracker	0.5
Surimi	1.8
Spring Roll	1.3

The limit of quantification (LOQ) of the **SENSI*Spec* ELISA Fish (Parvalbumin) test** is 4 ppm.
As matrices can have variable influence on the LOD in specific cases, and the range of matrices that was tested is of course limited, the end user if needed may evaluate its own LOD values depending on the matrices to be analyzed.
Alternatively, any results below LOQ should be just reported quantitatively as "< LOQ".

11.2 Precision

Intra-assay Precision	7-12%
Inter-assay Precision	4-10%

11.3 Linearity

The serial dilution of spiked samples (Wine, soup, Worcester sauce, asia sauce, cracker, surimi and spring roll) resulted in a dilution linearity of 89–105%.

11.4 Cross-reactivity

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

Almond	Hazelnut	Pork
Barley	Lamb	Potato
Bean	Macadamia	Pumpkin seed
Beef	Milk	Rice
Brazil nut	Millet	Rye
Buckwheat	Mustard	Scallop
Carrot	Oat	Sesame
Cashew	Onion	Shrimp
Celery	Pea	Soy
Corn	Peanut	Sunflower seed
Egg	Pecan	Walnut
Fish gelatin	Pistachio	Wheat

The following cross-reactions were determined:

Chicken	0.0004%
Isinglass	0.0005%
Squid	0.001%

11.5 Recovery

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

Wine (red)	103%
Soup	117%
Worcester Sauce	112%
Asia Sauce	103%
Cracker	99%
Surimi	114%
Spring Roll	93%

12. REFERENCES

1) Faeste CK, et al. (2008) – Quantitative sandwich ELISA for the determination of fish in foods. J Immun Methods, 329(1-2):45-55
2) Weber P, et al. (2009) – Competitive indirect ELISA for the determination of parvalbumins from various fish species in food grade fish gelatins and isinglass with parv-19 anti-parvalbumin antibodies. J Agric Food Chem, 57(23): 11328-11334
3) Gajewski KG, et al. (2009) – Monoclonal antibody specific to major fish allergen: parvalbumin. J Food Protect, 72(4):818-825
4) Chen L, et al. (2006) . Detecting fish parvalbumin with commercial mouse monoclonal anti-frog parvalbumin IgG. J Agric Food Chem, 54(15): 5577-5582
5) Swoboda I, et al. (2002) – Recombinant carp parvalbumin, the major cross-reactive fish allergen (…). J. Immun, 168 :4576-4584
6) Griesmeier U, et al. (2009) – Expression levels of parvalbumins determine allergenicity of fish species. Allergy, 65(2):191-198
7) Lim DLC, et al. (2008) – Parvalbumin – the major tropical fish allergen. Ped All Imm, 19(5):399-407
8) Lifrani A, et al. (2009) – Development of animal models and sandwich-ELISA tests to detect the allergenicity of fining agent residues in wines, J Agric food Chem, 57(2):525-534
9) Chatterjee U, et al. (2006) – Changes in the allergenicity during different preparations of pomfret, hilsa, bhetki and mackerel fish as illustrated by enzyme-linked immunosorbent assay and immunoblotting. In Arch allergy Immunol, 141(1):1-10
10) Van Do T, et al. (2005) – Allergy to fish parvalbumins: studies on the cross-reactivity of allergens from 9 commonly consumed fish. J Allergy Clin Immun, 116(6):1314-1320

13. LIABILITY

Gold Standard Diagnostics Budapest shall not be liable for any damages to the customer caused by the improper use of the kit and for any action undertaken as a consequence of the results.
Gold Standard Diagnostics Budapest shall not be liable for the unsafe use of the kit out of the current European safety regulations.