AlerTox® ELISA Fish Kit

For the quantitative detection of fish proteins in food products

REF KIT3060 (96 reactions)

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1. Introduction

The prevalence of fish allergies varies by location and ranges between 0 and 7%. Approximately 40% of fish allergies first appear in adulthood. In addition, fish allergies can sometimes be mistaken for similar adverse reactions to histamine produced by bacteria commonly found on improperly stored fish.

Fish muscles typically contain 13 – 20% protein. Although there are at least 12 allergenic fish proteins, the most common one is β-parvalbumin, which is heat stable and higher in content in white muscle than red muscle. Because symptoms can range from mild to severe, accurate and reliable detection of fish protein is important for consumer safety and compliance with food labeling regulations. Fish is categorized as one of the "Big 9" food allergens in the US.

If needed, the AlerTox ELISA® Histamine Kit is available for quantitation of histamine in fish (and wine) samples.

Note: Read this manual carefully before starting the test. The test must be performed by thoroughly trained staff.

1.1 Test Sensitivity and Specificity

The AlerTox ELISA Fish Kit detects and quantifies fish protein (parvalbumin) in fresh or lightly processed food products. The limit of detection (LOD) varies by matrix. For more details about the LOD, limits of quantification (LOQ) and expression of results, see the next table, *Section 4, Results Calculations* and *Section 6.2.1, Summary of Specifications*.

	AlerTox ELISA Fish Kit*
Limit of Detection (LOD)	1.4 ppm
Limit of Quantification (LOQ)	4 ppm
Quantification Range	4 – 100 ppm

* ppm = mg of fish meat (cod) per L or kg of sample.

The cross-reactivity with other food matrices is shown in the following table:

Cross-Reactive Matrix	Percent Cross-Reactivity (%)
Squid	0.001
Isinglass	0.0005

See *Section 6.2.2, Recovery* and *Section 6.2.3, Non-Cross Reactivity*, for additional data.

Important: Do not modify the protocol with respect to the timing, temperatures, plate washing, pipetting volumes, types of buffers or pH values of the buffers. Any of these protocol modifications will invalidate the test system.

1.2 Sample Preparation

Important: Please follow these instructions carefully, as there are sample preparation differences compared to most of the AlerTox ELISA Kits. The resulting sample extracts can only be tested using the AlerTox ELISA Fish test.

See *Section 6.1, Sample Extraction Compatibility*, for more details about other AlerTox ELISA Kits.

1.3 Test Principle

The AlerTox ELISA Fish Kit works on the principle of a quantitative sandwich ELISA. The antigen concentration is directly proportional to the color intensity of the test sample. Here is a brief overview of the sandwich ELISA test:

1. Primary antibodies directed against parvalbumin are bound on the surface of a microtiter plate. Parvalbumin-containing standards or test samples are placed into the wells of the microtiter plate. After a 20-minute incubation at room temperature (15 to 25 °C, 59 to 77 °F), the wells are washed with washing solution to remove unbound material.
2. Peroxidase-conjugated secondary antibodies directed against parvalbumin are put into the wells, and after a second 20-minute incubation, the plate is washed again.
3. The Substrate Solution is added, and the plate is incubated for another 20 minutes, resulting in the development of a blue color in positive wells. The addition of the Stop Solution inhibits further color development, and the color turns yellow. The yellow color is measured photometrically at 450 nm ($OD_{450\ nm}$).

2. Materials and Storage

2.1 Materials Supplied in the Kit

Item	Description	96 wells
1	Breakable strips of 8 wells, each coated with anti-parvalbumin primary antibodies. In a re-sealable foil bag containing a frame and drying agent. Ready to use.	12 strips
2	5 AlerTox Fish Standards, concentrations: 0 – 4 – 20 – 50 – 100 ppm. Ready to use.	5 x 3 mL
3	Conjugate: Peroxidase-conjugated, anti-parvalbumin secondary antibodies. Ready to use.	1 x 15 mL
4	Substrate Solution, containing trimethylbenzene (TMB). Ready to use.	1 x 15 mL
5	Stop Solution, containing sulfuric acid (H_2SO_4). Ready to use.	1 x 15 mL
6	10X Extraction & Sample Dilution Buffer.	4 x 30 mL
7	10X Washing Solution.	2 x 60 mL

2.2 Storage Conditions and Stability

- All kit components should be kept at 2 to 8 °C (36 to 46 °F) in the dark. DO NOT FREEZE.
- Return all reagents to 2 to 8 °C (36 to 46 °F) immediately after use.
- The diluted Washing Solution (1X) can be used for 4 weeks when stored at 2 to 8 °C (36 to 46 °F).

  Important: If needed, redissolve precipitants by warming the 10X Washing Solution at 37 °C (99 °F) for 15 minutes before dilution. Do not use the buffer if the precipitant does not redissolve.
- The diluted Extraction & Sample Dilution Buffer (1X) can be used for 1 week when stored at 2 to 8 °C (36 to 46 °F).

  Important: If needed, redissolve precipitants by warming the 10X Extraction & Sample Dilution Buffer at 37 °C (99 °F) for 15 minutes before dilution. Do not use the buffer if the precipitant does not redissolve.
- The Sample Extracts are stable for at least 24 hours at 2 to 8 °C (36 to 46 °F) or longer when frozen.

2.3 Material Required but Not Provided

- AlerTox Polyphenol Additive (Product No. ASY3213), only for samples with polyphenols and antioxidants*
- Multi-channel pipettor: 50 – 200 µL
- Sterile pipette tips
- Pipettors: 10 – 100 µL, 100 – 1,000 µL
- Water bath, adjustable to 60 °C (140 °F)
- 15 – 30 mL containers for the extractions
- ELISA Plate Reader with filter (450 nm) (Absorbance 96 ELISA Reader, Product No. MCH3005, or similar)
- Centrifuge
- Distilled water
- Stomacher, Mill, Mortar, Blender, etc.
- Vortex mixer

* Examples of foods rich in polyphenols, including tannins, and antioxidants are chocolate, tea, coffee, wine, purple corn and corn fiber, soy, berries and legumes, such as chickpeas or lentils.

2.4 Optional Materials/Equipment

- Homogenizer for sample extraction
- Repeating pipettor to minimize assay drift
- *Recommended*: An ELISA plate washer system to reduce the washing time and improve consistency

AlerTox ELISA Kits have been validated on fully automated ELISA systems (such as the BEAR Automated ELISA Robot). For validation details, contact us at www.hygiena.com/support.

3. Test Procedure

3.1 Reagent Preparation

We advise preparing reagents immediately before use and only preparing the amount necessary for the number of samples plus the 5 standards. Duplicate measurements of each sample and standard are recommended based on good laboratory practices (GLP) and quality control requirements.

Important: All reagents must be at room temperature (15 to 25 °C, 59 to 77 °F) at the time of use.

3.1.1 Extraction & Sample Dilution Buffer

Dilute the 10X Extraction & Sample Dilution Buffer 1:10 with distilled water to create the 1X solution.

Important: If needed, redissolve precipitants by warming the 10X Extraction & Sample Dilution Buffer at 37 °C (99 °F) for 15 minutes before dilution. Do not use the buffer if the precipitant does not redissolve.

Note: You will need the following amounts for each sample in your test:

Sample Type	Amount of Sample	Amount of 1X Extraction & Sample Dilution Buffer
Solid	0.5 g	10 mL
Liquid	0.5 mL	9.5 mL

3.1.2 Washing Solution

Dilute the 10X Washing Solution 1:10 with distilled water to create the 1X solution.

Important: If needed, redissolve precipitants by warming the 10X Washing Solution at 37 °C (99 °F) for 15 minutes before dilution. Do not use the buffer if the precipitant does not redissolve.

Note: You will need approximately 2.5 mL of 1X Washing Solution per well.

3.1.3 ELISA Plate

To prepare the ELISA plate, open the foil bag, remove the number of strips required to run the tests (samples plus the 5 standards, all in duplicate) and put the strips into a frame.

Notes:

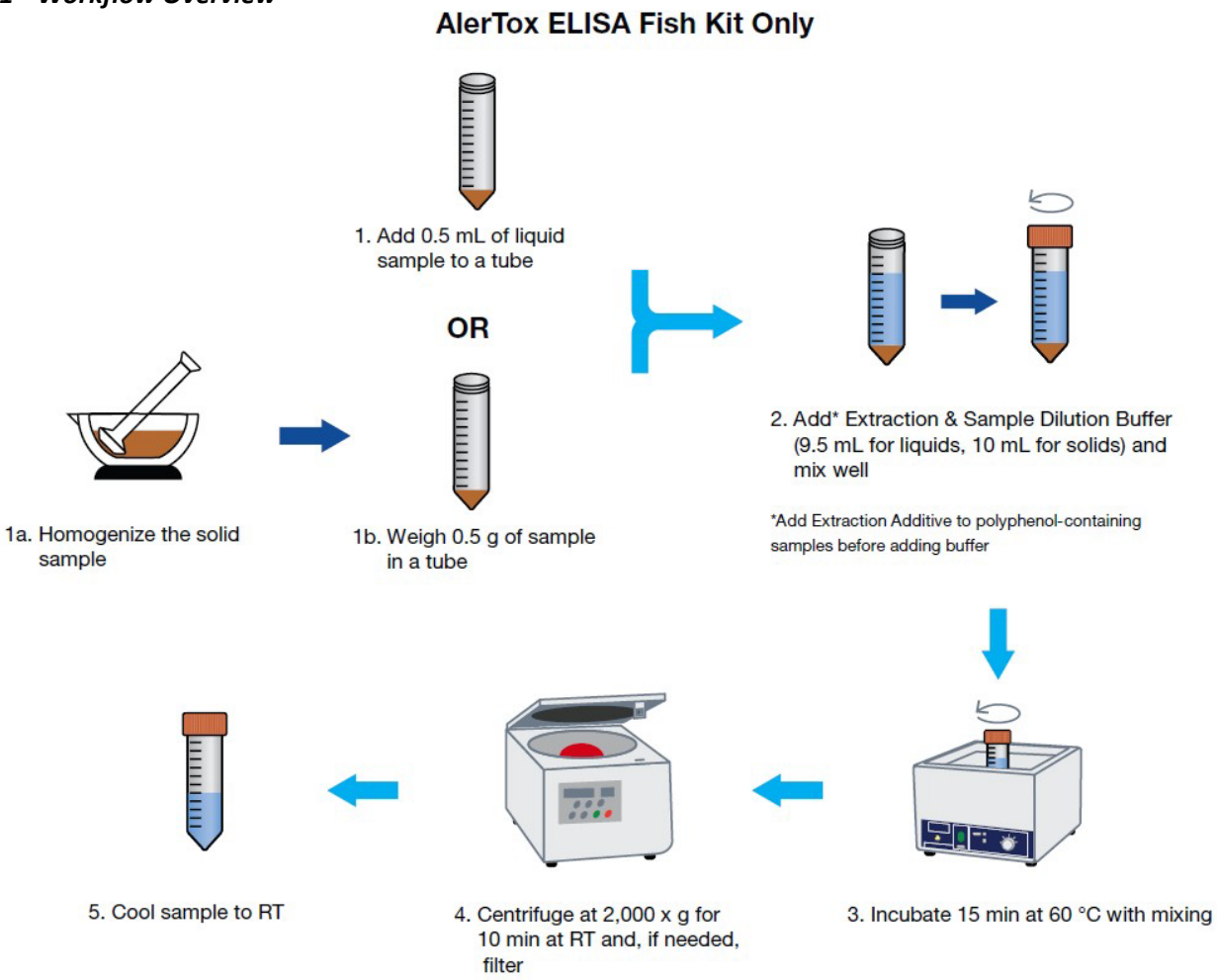
- When opening the foil bag for the first time, be careful not to cut the ziplock off the bag.
- Unused wells must be stored in the foil bag with the drying agent at 2 to 8 °C (36 to 46 °F). Ensure the ziplock on the foil bag is sealed tightly.

3.2 Sample Preparation

Important: See *Appendix A* for the sample preparation protocol for samples containing polyphenols, tannins or antioxidants. For other samples, follow the procedure below:

1. Resuspend sample in 1X Extraction & Sample Dilution Buffer based on sample type:
    a. For solid samples:
        i. Maximize the homogeneity of the sample by finely pulverizing a minimum of 5 g of sample in a mortar, impact mill or a similar device.
        ii. Resuspend 0.5 g of the homogenized mixture in 10 mL of 1X Extraction & Sample Dilution Buffer.
    b. For liquid samples:
        i. Add 0.5 mL of the liquid sample to 9.5 mL of 1X Extraction & Sample Dilution Buffer.
2. Mix well.
3. Incubate the mixture for 15 minutes in a preheated water bath at 60 °C (140 °F), shaking samples every 2 minutes to ensure homogeneity.
4. Centrifuge the mixture for 10 minutes at 2,000 x *g* at room temperature (15 to 25 °C, 59 to 77 °F). If the supernatant is still not completely separated from the precipitate, filter the supernatant.
5. Cool the sample extract (supernatant or filtrate) to room temperature (15 to 25 °C, 59 to 77 °F).

3.2.1 Workflow Overview



Important: Sample preparation for this kit is different from other AlerTox ELISA Kits.

3.3 ELISA Procedure

Important: The most critical points of the ELISA procedure are the temperature, timing and plate washing. Insufficient washing will result in poor precision and false results.

Note: For higher reproducibility, we recommend using a well-maintained, automated plate washer in steps 3 and 6 below.

1. Add 100 µL of the standards or prepared sample extracts in duplicate into the appropriate wells of the microtiter plate.

   Note: See *Section 7, Example Assay Layout*. If you have a large number of samples, pipette one set of standards before the samples and the duplicate set of standards after the samples and use the arithmetic mean values for calculations.

2. Incubate for 20 minutes at room temperature (15 to 25 °C, 59 to 77 °F).

   Important: Do not shake the plate during this incubation.

3. Wash plates **three (3)** times with 300 µL of 1X Washing Solution per well.

   Note: At the end of the automated washing or between each manual wash, invert the plates and strike them against clean, dry paper towels to empty the wells and remove residual liquid.

4. Add 100 µL of Conjugate Solution into each well.
5. Incubate for 20 minutes at room temperature (15 to 25 °C, 59 to 77 °F).

   Important: Do not shake the plate during this incubation.
6. Wash plates **five (5)** times with 300 µL of 1X Washing Solution per well.

   Note: At the end of the automated washing or between each manual wash, invert the plates and strike them against clean, dry paper towels to empty the wells and remove residual liquid.
7. Pipette 100 µL of Substrate Solution into each well.
8. Allow the reaction to develop in the dark (the substrate is light-sensitive) for 20 minutes at room temperature (15 to 25 °C, 59 to 77 °F).

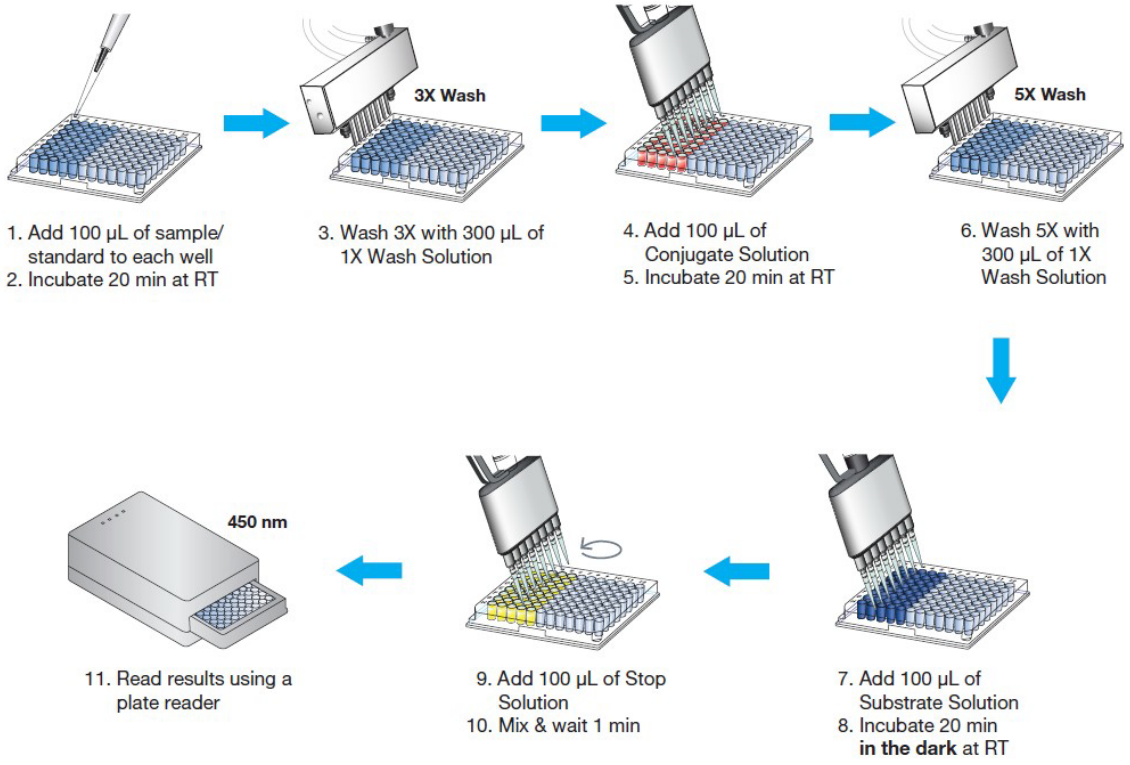
   Important: Do not shake the plate during this incubation.
9. Stop the enzyme reaction by adding 100 µL of Stop Solution (0.5 M H_2SO_4) into each well.
10. Gently shake the plate by hand and wait for 1 minute.

    Note: Wells containing blue color turn yellow in the presence of parvalbumin.
11. To measure results, use an ELISA plate reader with a 450 nm filter ($OD_{450\ nm}$), following the instrument manufacturer's instructions.

    Notes: Measure the color change within 30 minutes.

    Important: If any sample results fall outside the range of the fish meat standard curve, do not extrapolate the data. Instead, dilute the sample extract further with 1X Extraction & Sample Dilution Buffer and repeat the ELISA test using this diluted sample extract and standards, in duplicate.

3.3.1 Workflow Overview



4. Results Calculations

The results are measured as the concentration of whole fresh fish (cod) and not as parvalbumin. See Step 5 below for conversion factors to calculate the concentrations of other fish species.

The standards are prepared for a direct determination of fresh fish (cod) concentrations in samples. The dilution of samples in the extraction process, as described in the sample preparation procedures, is already taken into consideration when calculating levels. However, results must account for any additional dilution (e.g., due to high sample concentration or some alternative sample extraction procedures) (Step 4, notes below). Use the *AlerTox ELISA Calculation Worksheet* (available at www.hygiena.com/documents) or the following instructions to calculate results.

Important: Do not use the *AlerTox ELISA Calculation Worksheet* if the Zero Standard on the plate reader software is defined as the Blank for the calculation of $B - B_0$.

When interpreting the results, the arithmetic mean is used for calculations.

1. Calculate the mean OD value ($OD_{450\ nm}$) for each set of duplicate reference standards and duplicate samples.
2. Subtract the mean value of the Zero Standard from each mean OD value of standards or samples ($OD - OD_{Standard\ 0} = B - B_0$). See below, *Example Assay Data*.

   Important: If the Zero Standard on the plate reader software is defined as the Blank for the calculation of $B - B_0$, skip this step.
3. To create the standard curve, plot the adjusted OD values of standards 1 to 4 on the y-axis versus the concentration of whole fresh fish (cod) in ppm on the x-axis. See below*, Example of a Typical Standard Curve*.
4. For each sample extract, find the value $B - B_0$ on the y-axis. Then, read the corresponding value on the x-axis of the standard curve to determine the concentration of whole fish (cod).

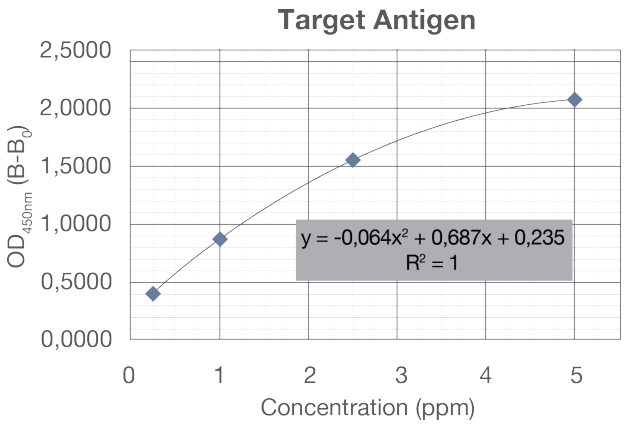
   Note: When using the standard sample preparation procedure (Section 3.2), it is not necessary to multiply the resulting concentration of the foodstuff sample by the dilution factor of 20.
5. To convert ppm of cod to ppm of another fish species, multiply the results by a species-specific conversion factor:

Fish Species	Conversion Factor (Multiply by)	Fish Species	Conversion Factor (Multiply by)
Bass	5.0	Red mullet	7.5
Carp	2.6	Red snapper	29
Catfish	1.7	Redfish	103
Devilfish	274	Salmon	1.7
Eel	29	Samlet	1.7
Flounder	7.1	Sardine	101
Haddock	21	Shark catfish	4.2
Hake	12	Spined loach	32
Herring (smoked)	13	Swordfish	1,250
Mackerel (smoked)	50	Trout	2
Perch	5.2	Tuna	370
Pike	0.3	Turbot	27
Plaice	2.4	Zander	11

Example Assay Data

Standard	Target Antigen [ppm]	Mean OD_{450nm}	$B - B_0$
Zero	0.0	0.108	—
1	2.0	0.265	0.157
2	10.0	0.606	0.498
3	25.0	1.193	1.085
4	50.0	1.928	1.820

Example of a Typical Standard Curve



5. General Precautions

- If your skin comes in contact with toxic or irritating substances, rinse the affected area with plenty of water and seek medical attention if needed. Please refer to the SDS, available at www.hygiena.com/SDS.
  - The Substrate Solution contains TMB, which is highly toxic if inhaled, ingested or contacts skin. Please refer to the SDS.
  - The Stop Solution contains H_2SO_4, which is corrosive. Please refer to the SDS.
- Handle the test kit in accordance with GLP.
  - Do not use reagents beyond the expiration date of the kit.
  - Handle all solutions with gloves.
  - During the sample extraction, avoid cross-contamination.
  - Devices, such as a blender, must be cleaned after each sample preparation.
  - Use sterile pipette tips.
  - Do not exchange reagent vial caps.
  - Do not interchange reagents between kits of different lot numbers.
- Do not alter reagents. Doing so can cause inaccurate results.
- All reagents must be equilibrated to room temperature (15 to 25 °C, 59 to 77 °F) before use.
- Do not use solutions if they become cloudy or precipitate. The only exceptions are 10X Washing Solution and 10X Extraction and Sample Dilution Buffer, which may have crystalline precipitants that must be completely dissolved before use (see Section 2.2).
- Substrate Solution is light sensitive. Avoid exposure to direct light and store in the dark.
- Use only distilled water for the dilution of concentrated buffers.
- Do not allow wells to dry completely.
- Avoid incubating microtiter plates on cold work benches.

6. Additional Information

6.1 Sample Extraction Compatibility

Individual samples must be extracted separately when using the following kits:

Individual Sample Extractions Required		
AlerTox ELISA Casein	AlerTox ELISA Crustacean	AlerTox ELISA Fish
AlerTox ELISA Histamine*	AlerTox ELISA Lysozyme†	AlerTox ELISA Milk

* The AlerTox ELISA Histamine Kit is based on a competitive ELISA test, while all other AlerTox ELISA Kits are based on sandwich ELISA tests.

† Cheese and other food samples, except for wine, must be extracted separately.

The following AlerTox ELISA kits share the same sample preparation protocol, meaning the sample extract can be tested using 16 different ELISA Assays:

Compatible Sample Extractions			
AlerTox ELISA Almond	AlerTox ELISA BLG*	AlerTox ELISA Cashew	AlerTox ELISA Coconut
AlerTox ELISA Egg	AlerTox ELISA Hazelnut	AlerTox ELISA Lupine	AlerTox ELISA Lysozyme†
AlerTox ELISA Macadamia	AlerTox ELISA Mustard	AlerTox ELISA Ovalbumin	AlerTox ELISA Peanut
AlerTox ELISA Pistachio	AlerTox ELISA Sesame	AlerTox ELISA Soy (STI‡)	AlerTox ELISA Walnut

* BLG = β-lactoglobulin

† Only the wine extract is compatible. (Cheese and other food extracts are not compatible.)

‡ STI = Soy trypsin inhibitor

6.2 AlerTox ELISA Fish Kit

6.2.1 Summary of Specifications

Specification	AlerTox ELISA Fish*
Results	Concentration of fresh fish meat (cod)
Limit of Detection (LOD)	1.4 ppm
Limit of Quantification (LOQ)	4 ppm
Standard Range	0 – 100 ppm
Quantification Range	4 – 100 ppm

* ppm = mg of fresh fish meat (cod) per kg of sample

Specification, continued	AlerTox ELISA Fish			
Calculation Factors†	*Fish*	*Multiply by*	*Fish*	*Multiply by*
	Bass	5.0	Red mullet	7.5
	Carp	2.6	Red Snapper	29
	Catfish	1.7	Redfish	103
	Devilfish	274	Salmon	1.7
	Eel	29	Samlet	1.7
	Flounder	7.1	Sardine	101
	Haddock	21	Shark catfish	4.2
	Hake	12	Spined loach	32
	Herring (smoked)	13	Swordfish	1,250
	Mackerel (smoked)	50	Trout	2
	Perch	5.2	Tuna	370
	Pike	0.3	Turbot	27
	Plaice	2.4	Zander	11

† Use the calculation factor to convert the results to the concentration of different types of fish.

For lot-specific assay data and acceptance/rejection criteria for measured values, see the Certificate of Analysis (www.hygiena.com/COA).

6.2.2 Recovery

Matrix*	Recovery (%)
Asia sauce	103
Cracker	99
Red wine	103
Spring roll	93
Soup	117
Surimi	114
Worcestershire sauce	112

* Tested in typical matrices.

6.2.3 Non-Cross Reactivity

Of the matrices that were tested, the following were found to be non-cross-reactive with the AlerTox ELISA Fish Kit:

Non-Cross-Reactive Matrices				
Almond	Barley	Bean	Beef	Brazil nut
Buckwheat	Carrot	Cashew	Celery	Chicken
Corn	Egg	Gelatin, fish	Hazelnut	Lamb
Macadamia	Milk	Millet	Mustard	Oat
Onion	Pea	Peanut	Pecan	Pistachio
Pork	Potato	Pumpkin seed	Rice	Rye
Scallop	Sesame	Shrimp	Soy	Sunflower seed
Walnut			Wheat	

7. Example Assay Layout

S0: Zero Standard (without antigen): the mean value = B_0.

S1 – S4: Standards: the mean value = B.

SP: Samples: the mean value = B.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**S0**	**S0**	SP4	SP4	SP12	SP12						
B	**S1**	**S1**	SP5	SP5	Etc.	Etc.						
C	**S2**	**S2**	SP6	SP6	Etc.	Etc.						
D	**S3**	**S3**	SP7	SP7	Etc.	Etc.						
E	**S4**	**S4**	SP8	SP8	Etc.	Etc.						
F	SP1	SP1	SP9	SP9	Etc.	Etc.						
G	SP2	SP2	SP10	SP10	Etc.	Etc.						
H	SP3	SP3	SP11	SP11	Etc.	Etc.						

8. Disclaimer

Field of use: Use the Hygiena product for research and development, quality assurance and quality control under supervision of technically qualified persons. The information generated from the Hygiena product is only to be used in conjunction with the user's regular quality assurance program. The Hygiena product should not be used as the sole basis for assessing the safety of products for release to consumers. Data obtained from the Hygiena product must not be used for human diagnostic or human treatment purposes. Before using product, read the *Limitation of Warranty and Liability* (available in the *Hygiena General Terms and Conditions* at www.hygiena.com/terms-and-conditions).

These products are made from high-quality raw materials. No warranty of any kind is made, either expressed or implied, as to their suitability other than to measure the target antigen content when used exactly in accordance with these instructions, except regarding the quality of these materials.

Use of the kit for any other purpose is outside its intended use. For matrices that have not been previously validated, Hygiena cannot guarantee that the kit is fit for purpose and that the results obtained for these matrices are accurate. Customers may choose to use the product on unvalidated food or surface matrices; however, Hygiena strongly recommends that users perform their own fit-for-use testing to confirm suitability and performance in their specific application. Any damages, including consequential or special damage or expense arising directly or indirectly from using this product, are limited to the replacement value of the kit.

For additional information or assistance with matrix validation, contact Hygiena at www.hygiena.com/support. All Hygiena Terms and Conditions apply and can be found at: www.hygiena.com/terms-and-conditions.

9. Contact Information

For more information, visit www.hygiena.com/contact. For technical support, visit www.hygiena.com/support.

10. Change Index

INS3022 REVD, July 2020
Clarified parts of the conversion factors table.

INS-KIT3060-001-REVA, June 2025
Updated recovery data, selectivity data and document ID number. Included use of the AlerTox Polyphenol Additive for some sample preparations.

Appendix A. Specialized Sample Extraction Procedures

A.1 For Foods and Drinks Containing Polyphenols, Tannins or Antioxidants

Follow this sample preparation protocol when testing foods and drinks that are rich in polyphenols, including tannins, and antioxidants. Examples are listed in the following table:

Representative Matrices		
Berries	Chocolate	Corn, purple
Corn fiber	Coffee	Legumes (e.g., chickpeas, lentils)
Soy	Tea	Wine

Important: This procedure is **not** for use with the following kits:

- AlerTox ELISA Crustacean Kit
- AlerTox ELISA Histamine Kit
- AlerTox ELISA Lysozyme Kit
- Wine extracts for the following kits:
  - AlerTox ELISA Casein Kit
  - AlerTox ELISA Ovalbumin Kit

a. For solid samples, maximize the sample homogeneity by finely pulverizing a minimum of 5 g of sample in a mortar, impact mill or similar device.

Note: For liquid samples, proceed to Step b.

b. Mix the sample with the AlerTox Polyphenol Additive (Product No. ASY3213) and 1X Extraction & Sample Dilution Buffer, based on the kit used:

i. For AlerTox ELISA Kits except Hazelnut and Pistachio: mix the sample and AlerTox Polyphenol Additive first, then add 1X Extraction & Sample Dilution Buffer (see table below) and mix well.

ii. For the AlerTox ELISA Hazelnut and Pistachio Kits: Dissolve 1 g of AlerTox Polyphenol Additive in 100 mL of 1X Extraction & Sample Dilution Buffer before mixing with the specified amount of sample (see table below).

Kit	Sample	AlerTox Polyphenol Additive	1X Extraction & Sample Dilution Buffer
AlerTox ELISA Kits*	1 g (Step a, solid)	2 g	20 mL
	1 mL	2 g	19 mL
AlerTox ELISA Milk Kit	0.5 g (Step a, solid)	1 g	10 mL
	0.5 mL	1 g	9.5 mL
AlerTox ELISA Hazelnut and Pistachio Kits	0.5 g (Step a, solid)	10 mL	
	0.5 mL	9.5 mL	

* i.e., all AlerTox ELISA Kits except those specific for hazelnut, pistachio, milk or those excluded in the Important note above.

c. Incubate for 15 minutes in a preheated water bath at 60 °C (140 °F), shaking the samples every 2 minutes to ensure homogeneity.

d. Centrifuge for 10 minutes at ≥ 2,500 x *g*.

e. If the supernatant is still not completely separated from the particulates, filter the supernatant.

f. Proceed with the *ELISA Procedure* (Section 3.3).

Important: The results calculations will **not** require additional dilution-factor adjustments for this procedure.

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