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Product Manual

AgraQuant® Fish ELISA test kit
Article number 10002083

Intended use
The AgraQuant® Fish ELISA test kit is an immunoassay designed for the quantitative analysis of fish parvalbumin residues in food samples. Samples can vary from raw to processed foods, from environmental swabs to rinse water. This product is intended for laboratory use.

Minimum performance characteristics

Limit of detection (LOD): 1.4 ppm (1.4 mg/kg) fish (cod)

Limit of quantification (LOQ): 4 ppm (4 mg/kg) fish (cod)

Range of quantification: 4 – 100 ppm (4 - 100 mg/kg) fish (cod)

Plate format: 48 wells

Assay time: sample preparation – 25-30 minutes (approx.)
total incubation time – 60 minutes

About Fish
Part of the human diet for thousands of years, fish is considered important for a healthy diet due to its high content of unsaturated fatty acids. However, in regions like Scandinavia, Japan or the Mediterranean area where the consumption of fish is high, allergy to fish is quite common and represents a serious health concern. In most cases, fish allergy is caused by the calcium-binding protein parvalbumin, a major allergen. This protein is highly resistant to heat and is very stable against denaturing agents and proteolytic enzymes.
For people suffering from a fish allergy, hidden fish proteins in food are a critical problem. Any intake of fish must be strictly avoided to prevent potentially fatal allergic reactions. Fish and fish-derived products are regularly used in the food industry. Furthermore, potential cross-contamination with fish during food production process cannot always be prevented. Thus, fish represents a genuine threat for allergic individuals. The detection of fish in food products and production lines is therefore crucial.

IMPORTANT: this version introduces major changes in the cross-reactivity values and conversion factors. This document is valid from Lot no. 1000002178 on.

Product information

About the ELISA test kit

The AgraQuant® Fish test kit is an enzyme-linked immunosorbent assay (ELISA) sandwich used for the quantification of fish in food samples. This product is a very sensitive detection system and utilizes highly purified polyclonal antibodies raised against fish parvalbumin for the quantification of fish residues and traces in a variety of food products. With AgraQuant® Fish, raw materials and finished food samples can be tested for the presence of fish. AgraQuant® Fish can also be used to validate cleaning procedures and to test for the presence of allergenic traces via rinse waters or environmental swab.

Storage information

Always store the AgraQuant® Fish ELISA Test kit at 2-8°C (35-46°F) when not in use. Do not freeze. Do not use the kit beyond the expiration date indicated on the package.

Content of the kit

The AgraQuant® Fish ELISA test kit contains the following items:

- 48 antibody-coated microwells (6 eight-well strips) in a microwell holder, sealed in a foil pouch
- 5 vials of 4 mL of ready-to-use standard solutions (0, 4, 10, 40 and 100 ppm of cod)
- 1 green-capped bottle of 7.5 mL of enzyme-conjugate solution (detection antibody)
- 1 blue-capped bottle of 7.5 mL of substrate solution
- 1 red-capped bottle of 7.5 mL of stop solution
- 1 bottle of 120 mL of 10X concentrated extraction buffer
- 1 bottle of 60 mL of 10X concentrated wash buffer

Materials required but not included

Extraction Procedure:

- Blender, mortar and pestle, or homogenizer
- Analytical balance
- Graduated cylinder, 100 mL
- Distilled or deionized water for diluting concentrated buffers
- Container with a minimum capacity of 20 mL
- Water bath (60°C/140°F)
- Centrifuge, micro-centrifuge (or filter and funnel) and centrifuge tubes
- Vortex

Assay Procedure:

- Calibrated 8-channel and single-channel pipettes with 100 µL disposable plastic tips
- Timer
- Plate washer or wash bottle
- Distilled or deionized water
- Absorbent paper towels
- 3 reagent boats to be used as reagent containers for an 8-channel pipette
- Microwell reader with 450 and 630 nm filters

Visit **www.romerlabs.com** or get in touch with your technical sales representative to find out which of these items are also available from Romer Labs.

ELISA kit – Assay principle

AgraQuant® ELISA test kits are based on solid phase sandwich ELISA technology. The kit comes with antibodies raised against the allergen of interest pre-coated onto each microwell. During the first incubation, both samples and standards of known concentrations are added into the microwells to allow any allergen present in the sample to bind to the immobilized antibodies.
After washing, the enzyme-conjugated solution (detection antibody) is added to the microwells and binds to the allergens captured during the first incubation forming a "sandwich". After a second washing step to remove excess detection antibodies, the substrate solution is added, which results in color development. The intensity of the color developed is proportional to the concentration of allergen present in each microwell.
A stop solution is then added, which changes the color from blue to yellow. The absorbance of each well is measured at 450 nm with the differential filter set at 630 nm as reference. The measurement must take place within 10 minutes after adding the stop solution.
To analyze the results, please refer to Results analysis at the end of this product manual.

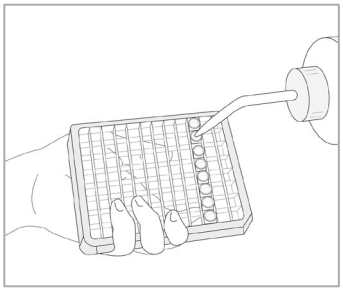
Protocol at a glance

The following section gives only an overview of the ELISA procedure. Before performing the assay, carefully read through this product manual.

1

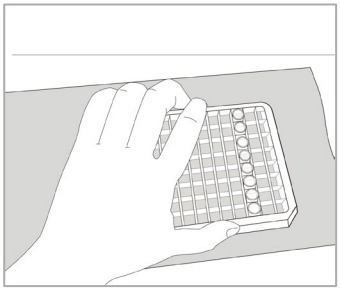
Dispense **samples** and **standards** into the antibody-coated wells. **Incubate for 20 min**

2



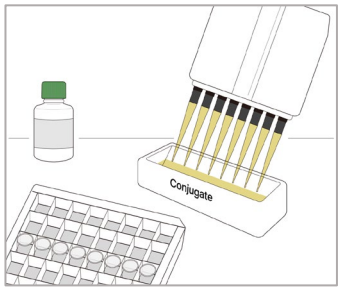
Carefully empty the mirowells and wash **5 times** with diluted wash buffer.

3



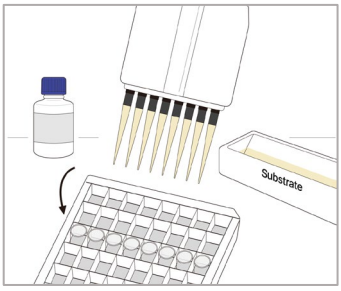
Tap the microwell strips onto absorbent paper towels to remove any residual buffer.

4



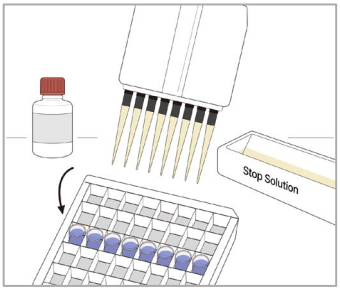
Pipette the **enzyme-conjugate solution** into the microwells.
Incubate for 20 min.
Wash as in steps 2 and 3.

5



Pipette the **substrate solution** into each well.
Incubate for 20 min in the dark.

6



Stop the reaction by pipetting the **stop solution** into each microwell.

7. Read the absorbance of each well at 450 nm (reference wavelength 630 nm) with an microwell (ELISA plate) reader. Calculate results according to the *Results Analysis* section.

Reagent and sample preparation

Buffer preparation

Extraction buffer:
Calculate the amount of extraction buffer needed according to the number of samples. Prepare extraction buffer by diluting the 10X concentrated extraction buffer 1:10 with distilled water (e.g., add 10 ml of concentrated extraction buffer to 90 mL distilled water to obtain a final volume of 100 ml extraction buffer). Heat to 60 °C using a water bath. Label as extraction buffer.
Note: the extraction buffer is stable for up to a week if stored at 2-8°C (35-46°F).

Wash buffer:
Dilute the 10X concentrated wash buffer 1:10 with distilled water (e.g., add 10 mL of concentrated wash buffer to 90 mL distilled water to obtain a final volume of 100 mL wash buffer). Label as diluted wash buffer.
Note: Diluted wash buffer is stable for four weeks if stored at 2- 8°C (35-46°F).

If crystals form in any of the concentrated buffers during cold storage, they should be warmed up to 37°C (98°F) until they dissolve.

Sample preparation

Solid samples:

1. Obtain a representative sample of the specimen you want to analyze and homogenize a minimum of 5 g in a mortar or blender.
2. Weigh **1 g** homogenate in a 50 mL centrifuge tube and add **20 mL pre-warmed extraction buffer**. Vortex until all the solid is suspended.
3. Shake the suspension for **15 minutes.**
4. Centrifuge samples for **10 minutes** at 2000 g to obtain a clear supernatant. If there still are particles in the supernatant, filter it and collect the filtrate.
   Note: If a centrifuge is not available, filter the extract with filter paper and collect the filtrate.
5. Samples are ready for testing. For each well, apply 100 µL of the particle-free sample extract obtained. Please read the *ELISA procedure* section and carefully follow the protocol.

Liquid samples:

1. Obtain a representative sample of the specimen you want to analyze.
2. Take a **1 mL** aliquot of the liquid sample into a 50 mL centrifuge tube and add **19 mL pre-warmed extraction buffer**. Vortex the solution/mixture.
3. Shake the suspension for **15 minutes.**
4. Centrifuge samples for **10 minutes** at 2000 g to remove any particles.
   Note: If a centrifuge is not available, filter the extract with filter paper and collect the filtrate.
5. Samples are ready for testing. For each well, apply 100 µL of the particle-free sample extract obtained. Please, read the *ELISA procedure* section and carefully follow the protocol.

Swab samples:
AgraQuant® Allergen ELISA test kits can be used to analyze environmental samples obtained with AgraQuant® Allergen Swabbing kit.
Carefully read and follow the instructions of the AgraQuant® Allergen Swabbing kit. Samples thus obtained are ready to use and do not require further extraction. They can be directly pipetted into the wells.
Different ranges of quantification apply for the swabs. For more details, please refer to the package insert of the AgraQuant® Allergen Swabbing Kit.

Notes:
If the sample is expected to be highly contaminated, further dilution can be carried out using extraction buffer. The additional dilution factor must be considered when calculating the final concentration. For more details, see section *Results Analysis*.

Some samples could give negative results, but still contain allergen below the limit of detection of the test kit. The food matrix of certain foods and high degree of processing can influence the results of the test.

The AgraQuant® Fish ELISA test kit uses its own extraction buffer. Do not use sample extracts obtained with this kit with any other allergen ELISA test kit. Sample extracts obtained with AgraQuant® ELISA test kits cannot be used with AgraQuant® Plus ELISA test kits.

Sample specifications and storage

Effect of pH: Performing the assay in a pH around 8 will lead to reliable results. Highly acidic or alkaline samples might lead to false positive or false negative results. If you suspect that your samples might have extreme pH values, please check the pH after sample extraction. Where needed, the pH may be adjusted by adding NaOH or HCl. This is only a recommendation and not a requirement of the method.

Effect of polyphenols: Some matrices may contain high percentages of tannins or other polyphenols. In this case, the addition of a binding agent to the extraction buffer, prior to the extraction, is recommended. Examples of matrices with high polyphenol content are buckwheat, chestnut flour, chocolate, cocoa, coffee, millet, spices among others. Contact your technical sales representative for more details.

Cross-reactivity: Chicken 0.0004%, Isinglass 0.0005%, Squid 0.001%. Values of cross-reactivity with other fish species were used to calculate species conversion factors. They can be found in the last page of this package insert.

Storage: Sample extracts can be stored at 2–8°C (35-46°F) for up to 24 hours before running the assay. If longer storage is required, extracts can be stored at -20°C (-4°F) for up to 2 weeks.
Always equilibrate the samples to room temperature and vortex them or mix them by shaking before applying them to the ELISA plates.

Technical support
Not sure if the test works with your specific samples or matrices? Let our longstanding experience in food allergen testing work for you. Contact our technical sales representative in your region to know more.

ELISA procedure

Before starting

Procedural guidelines:

- Make sure you have everything you need ready before starting the assay.
- All reagents and kit components must be equilibrated to room temperature, i.e., 18-30°C (64-86°F), before use.
- It is good laboratory practice to run standards and sample extracts in duplicates.
- Run a standard curve with each assay.
- Adhere to the incubation times stated in the procedure. Use of incubation times other than those specified may give inaccurate results.
- It is strongly recommended that the assay be performed with an 8-channel pipette.
- The wash procedure is critical and therefore must be performed accurately.
- Do not run more than 6 eight-well strips in one experiment when using an 8-channel pipette.

Precautions:

- Do not mix or interchange reagent lots from different kits lots.
- Due to the high risk of cross-contamination, all used instruments must be cleaned thoroughly before sample preparation and running the assay. Adhere to the instructions for test procedures.
- Cover or close all reagents when not in use.
- The stop solution contains acid. Avoid contact with skin or eyes. If exposed, flush with water.
- Wear protective gloves and safety glasses when using the kit.
- Dispose of all materials and containers properly after use.

Assay protocol

Optional Transfer well method:

1. Place an adequate number of **transfer wells** in a microwell strip holder and then add **150 µl of each ready-to-use standard and prepared sample** into them. Use a fresh pipette tip for each standard and sample. Make sure to empty the pipette tip completely each time.
2. Place an appropriate number of **antibody-coated microwells** in a microwell strip holder and, using an 8-channel pipette, transfer **100 µl of each ready-to-use standard and prepared sample** from the transfer wells to the corresponding antibody-coated microwells.

Continue with step 3 of the standard assay protocol.

Standard assay protocol:

1. Place an appropriate number of **antibody-coated microwells** in a microwell strip holder. Once the desired number of strips are removed from the foil pouch, reseal the bag and store it at 2-8°C (35-46°F) to preserve it.
2. Using a single channel pipette, add **100 µL of each ready-to-use standards and prepared samples** into the appropriate well. Use a fresh pipette tip for each standard or sample. Make sure to empty the pipette tip completely.

3. Incubate at room temperature for **20 minutes**.
   Note: Do not attempt to mix the content of the microwells by shaking the plate as this may cause well-to-well contamination.
4. **Washing:** Empty the content of the microwell strips into a waste container. Wash by filling each microwell with wash buffer (prepared as stated in the "Buffer preparation" section) and then discarding it again. Repeat this step 4 times, for a total of **5 washes**.
   Note: Take care not to dislodge the strips from the holder during the wash procedure.
5. After the fifth wash, lay several layers of absorbent paper towels on a flat surface and tap the microwell strips on top of them to remove any residual buffer.
   Note: Never insert absorbent paper directly into the wells.
6. Measure the required amount of enzyme-conjugate solution from the green-capped bottle (~120 µL/well or 1 mL/strip) and dispense it into a separate container (e.g., reagent boat). Pipette **100 µL of enzyme-conjugate solution** into each microwell with an 8-channel pipette.
7. Incubate at room temperature for **20 minutes**.
8. **Washing:** Perform the washing step as described above at steps 4 and 5.
9. Measure the required amount of substrate solution from the blue-capped bottle (~120 µL/well or 1 mL/strip) and dispense it into a separate container (e.g., reagent boat). Pipette **100 µL of the substrate solution** into each microwell with an 8-channel pipette.
10. Incubate at room temperature for **20 minutes** and allow the reaction to develop **in the dark** (e.g., cover completely, or carefully place in a cupboard or drawer).
11. Measure the required amount of stop solution from the red-capped bottle (~120 µL/well or 1 mL/strip) and dispense it into a separate container (e.g., reagent boat). Stop the reaction by pipetting **100 µL of stop solution** into each microwell by using an 8-channel pipette. The color will change from blue to yellow.
12. Read the absorbance of each well **within 10 minutes** after the addition of the stop solution at 450 nm (reference wavelength 630 nm) with a microwell reader. Carefully remove any air bubbles prior to reading the absorbance as they may affect the result.
    Note: Do not return unused reagents to their original bottles. Carefully note which rows/strips contain standards or samples during the assay.

Results analysis

The content of fish in your samples can now be determined. The ready-to-use standards at known concentrations allow for the determination of the concentration of the unknown samples.
Calculate the mean value of the optical density (OD) of the duplicates for each standard. Build a dose-response curve by plotting the mean OD obtained for each of the five standards (y-axis) against its concentration in ppm (x-axis). Determine the corresponding concentration of fish in ppm by interpolation from the standard curve using a point-to-point calculation. If you are using a software-based quantification system, the four-parameter logistic curve (4PL) is recommended.

Alternatively, results can be easily calculated using the **Romer Labs spreadsheet** that is provided free of charge upon request. With the Romer Labs spreadsheet you only need to insert the obtained OD values and the fish amounts in your samples are calculated automatically using a point-to-point curve fitting.
When working according to the *sample preparation* section described in this package insert, a **dilution factor of 20** is applied during sample extraction. This dilution factor of 20 is already taken into account, thus the allergen concentration can be read directly from the standard curve obtained.
If the amount of allergen contained in a sample exceeds the value of the highest standard (fish (cod)

>100 ppm), the sample extract should be further diluted by using extraction buffer and then re-analyzed by performing a new assay. The dilution should be done such that the diluted sample results are within the AgraQuant® Fish ELISA test kit range (4 - 100 ppm). The dilution factor of this additional dilution step must be applied when the result is calculated.
Notes: An OD value of less than 1.1 absorbance units for the highest standard may indicate the deterioration of reagents. If the percent coefficient of variation (%CV) of the duplicate readings of the standards or of the samples exceeds 20%, the result of your test might be inaccurate. It is recommended to repeat the assay.

Swab samples: When running a swab sample, you can calculate the concentration of your sample, by using the concentration you read off the curve, as in the following example:

Value for swab sample read off curve: 10 ppm
Multiply this value by 1000 to convert the units to ng/ml: 10 ppm X 1000 = 10000 ng/ml
Since there was no dilution performed during preparation nor extraction, divide this value by the dilution factor considered in the curve (20): 10000 ng/ml ÷ 20 = 500 ng/ml

The AgraQuant® Fish ELISA Test kit detects the amount of fish parvalbumin contained in your sample and expresses the results as amount of cod fish per kg of sample. To calculate the corresponding amount of other fish species, you can use the following conversion factors. They were experimentally calculated during validation experiments, using whole commodities (wet weight): *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.* If processed food is analyzed, the grade of processing needs to be taken into account (e.g., cod meat cooked for 20 minutes resulted in a 25% reactivity compared to fresh cod).

Visit **www.romerlabs.com** to find worldwide contact information.
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