

# Accurate Measurement of Walnut Allergen in Food Products Using Hygiena's AlerTox® ELISA Walnut Kit

A Summary of Fapas® Proficiency Testing Study 27344

## The Importance of How Walnut Protein is Detected in Food

Tree nut allergies have an estimated prevalence of 0.5 – 7.3%, varying by region and age. Walnuts, along with hazelnuts and cashews, are the most common cause of tree nut allergies with symptoms ranging from mild to life-threatening. As a result, food labeling requirements for tree nuts have been established in most regions of the world, although there is no formal detection threshold guidance.

The lack of definitive detection values complicates the creation of safety-first allergen testing programs by food manufacturers and processors. Setting the value too high can lead to false-negative results. In contrast, for food testing, detection of very low levels of allergens is desirable, whereas during environmental testing, setting the value too low can trigger unnecessary time-consuming recleanings that can cause potential product release delays. Thus, using appropriate techniques (e.g., ELISA, immunochromatography) and testing methods becomes a critical component of HACCP standard operating procedures to verify allergen-free food and effective cleaning processes as well as reduce risks of allergen exposure and cross-contamination.

At Hygiena®, we refer to the scientific expert panel (VSEP) guidance behind Australia's Voluntary Incidental Trace Allergen Labeling (VITAL). VITAL's 2019 allergen threshold guidance was developed from published and unpublished data on low-dose oral food challenges in the United States, Australia and the European Union. One way that we can help food manufacturers and processors determine an appropriate way to measure tree nut allergens is to participate in proficiency testing (PT) studies that include the use of our allergen tests, such as the AlerTox® ELISA Walnut Kit.

PT provides an independent assessment of laboratory performance and allows us to compare our results to those of laboratories worldwide. Taking part in proficiency testing gives insight into a laboratory's equipment, methods, brands of products and technical skills of the staff, allowing participants to improve or confirm the delivery of the expected quality of results. The overall analysis and interpretations of PT findings can also help guide testing strategies when selecting appropriate methods.

## Description of the AlerTox ELISA Walnut Kits

The AlerTox ELISA Walnut Kit is an immunosorbent assay designed for the quantitative determination of walnut in raw materials and final products. The kit is based on the ELISA sandwich technique that is often used to analyze substances found at very low concentrations. This method, combined with the high specificity and sensitivity of the antibody used in these tests, allows this kit to precisely quantify walnuts in all types of food and drinks (See Table 4 in the [Appendix](#)).

## Proficiency Testing Method

Testing rounds were organized by an independent organization (i.e., Fera Science) that sent out identical samples (Table 1) to 33 participants, analyzed results from 27 participants (82%) who submitted data by the closing date of the study and produced publicly available reports. As in all Fapas Food Chemistry PT studies, participants were not told how much allergen, if any, was contained in each sample before testing.

All participating labs were asked to indicate both qualitative and quantitative results of walnut presence, including the limits of detection of the kits. Results are not retractable. The participants tested samples using their standard operating procedures and method(s) of choice. In this study, two laboratories (001 and 022) used the AlerTox ELISA Walnut Kit. Note: Laboratory 001 is affiliated with Hygiena, while Laboratory 022 is not.

**Table 1. Test Materials Used in Fapas Food Chemistry Proficiency Testing Study 27344.**

Material no.	Matrix	Walnut spiked value
27344A	Bread	None
27344B	Bread	~20 mg/kg (ppm)

## Results

### Qualitative and Quantitative Tests

For the qualitative assessments, there was 100% consensus for detecting the presence or absence of walnut from the 19 laboratories submitting this data, including Laboratories 001 and 022 (Table 2).

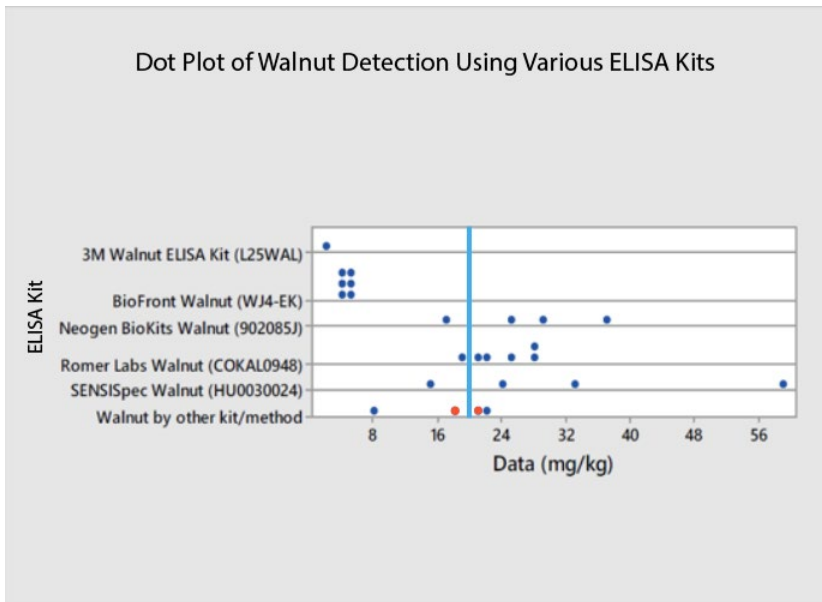
**Table 2. Fapas Proficiency Testing of Bread with Hygiena’s AlerTox ELISA Walnut Kit.**

Material no.	Qualitative results		Submitted quantitative value	
	Lab 001	Lab 022	Lab 001	Lab 022
27344A (no walnut)	Not detected	Not detected	<LOQ*	<2†
27344B (20 mg/kg walnut)	Detected	Detected	21.47 mg/kg	17.5 mg/kg

\* Limit of quantification (LOQ) = 2 ppm

† LOQ = 2 ppm

For sample 27344B, the measurements submitted by the two laboratories using the AlerTox ELISA Walnut Tests were both close to the spike-in value of 20 mg/kg (Table 2, Figure 1). For comparison, Figure 1 provides a summary of submitted values by kit for the study.



**Figure 1. Dot Plot of Walnut in Bread (Sample 27344B) by Kit.** 27344B was spiked with 20 mg/kg of walnut (vertical, light blue line). Each dot represents a submitted value from different participants. AlerTox ELISA Walnut Kit results from two laboratories are shown as red dots.

### Additional Analysis of Quantitation

As part of the analysis by Fera Science, results were separated into groups according to the ELISA kit used, and if there were enough samples, quantitative data were statistically analyzed to provide an assigned value based on the results submitted for each kit. Due to a low number of data points in this study, the median was chosen as the assigned value to represent consensus results for the three kits (Table 3). These median values were then used in combination with standard deviations to calculate a z-score for each result. The lower the z-score, the less variability in results. Any  $|z|$  value  $\leq 2$  is considered a ‘passing’ value. In this way, participants can compare how well their kits have performed to consistently measure the amount of allergen.

We calculated z-scores to compare our results using AlerTox ELISA Walnut to those from three other kits in this study (Table 3). The  $|z|$  values were  $\leq 2$  (pass; low variability) for results from the Romer Labs and Neogen kits, which had median values that were close to the spike-in amount. In contrast, the  $|z|$  value was  $>2$  (high variability) for results from the BioFront Technologies kit, which had a median value that was much lower than the spike-in amount.

**Table 3. Comparison of Results from AlerTox ELISA Walnut Kit with Three Other Kits.**

Result	Spike-in amount	Hygiena’s AlerTox ELISA Walnut Kit	Romer Labs’ AgraQuant® Walnut Kit	Neogen’s BioKits Walnut Assay Kit	BioFront Technologies’s MonoTrace® Walnut ELISA Kit
Value (mg/kg)	20	21.47 Laboratory 001	23.5 median	27.1* median	4.5 median
z-score	—	—	<b>-0.35</b>	<b>-0.83</b>	15.02

\* Statistical results from Neogen’s BioKits Walnut Assay Kit were provided for information only in the Fapas report.

### Conclusions

Hygiena’s AlerTox ELISA Walnut Kit performed equally well or better than other commercial kits included in this PT study. Measurements from two independent labs using the AlerTox ELISA Walnut Kit did not detect walnut in the unspiked bread sample and detected walnut in the spiked bread sample. Based on z-scores, the quantitative results from the AlerTox ELISA Walnut Kit were similar to those from Romer Labs or Neogen kits, which were generally accurate when viewed as dot plots. However, the quantitative results from the AlerTox ELISA Walnut Kit were not similar to those from the BioFront kit, which were visually more precise but not accurate on the dot plot.

With only 60 minutes of incubation time, this easy-to-use sandwich ELISA test has the sensitivity (Appendix Table 4) and specificity that makes it a useful part of a testing program for walnut allergen in a wide range of food matrices and beverages. The accurate detection and quantification of walnut in bread shown in this PT study supports our previous studies showing 103% recovery from cookies, 106% recovery from cornflakes and 87% recovery from ice cream. Additional recovery and cross-reactivity information is contained in the AlerTox ELISA Allergen instructions.

## Appendix: Additional Information About AlerTox ELISA Testing for Tree Nuts

Currently, six of our twenty AlerTox ELISA Kits target tree nuts (Table 4). Sample extracts prepared using the standard AlerTox ELISA protocol can be tested directly with any AlerTox ELISA tree nut assay, making it convenient to test for multiple tree nut allergens in the same sample.

**Table 4. Sensitivity of AlerTox ELISA Kits for Tree Nuts.**

AlerTox ELISA target	Limit of detection	Limit of quantification	Range of quantification
Almond	0.2 ppm	0.5 ppm	(10 – 5 – 2 – 0.5 – 0 ppm)
Cashew	0.2 ppm	2 ppm	(50 – 25 – 10 – 2 – 0 ppm)
Hazelnut	0.3 ppm	1 ppm	(40 – 15 – 5 – 1 – 0 ppm)
Pistachio	0.13 ppm	1 ppm	(25 – 12.5 – 5 – 1 – 0 ppm)
Macadamia	0.1 ppm	1 ppm	(25 – 12.5 – 5 – 1 – 0 ppm)
Walnut	0.6 ppm	2 ppm	(50 – 15 – 5 – 2 – 0 ppm)

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