Test Instruction

Walnut

96/48 Tests

Enzyme Immunoassay
for the Quantitative
Determination of
Walnut in Food

Cat.-No.: WAL-E01/E04

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

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Sensitivity (Walnut)	0.4 ppm
Recovery	60-106%
Incubation Time	60 min

General Information

Walnut (*Juglans regia*) belongs to the walnut plants (Juglandaceae). With 15% the fraction of proteins in walnuts is high. Some of these proteins are known for being allergenic, such as rJug r 1 and rJug r 4. Many of them are heat resistant making them stable to different production processes. For this reason walnut represents an important food allergen. For walnut allergic persons hidden walnut allergens in food are a critical problem. Already very low amounts of walnut can cause allergic reactions, which may lead to anaphylactic shock in severe cases. Because of this, walnut allergic persons must strictly avoid the consumption of walnuts or walnut containing food. Cross-contamination, mostly in consequence of the production process, is often noticed. The chocolate production process is a representative example. This explains why in many cases the existence of walnut residues in food cannot be excluded. For this reason sensitive detection systems for walnut residues in foodstuffs are required.
The **Eurofins Immunolab Walnut ELISA** represents a highly sensitive detection system and is particularly cap-able of the quantification of walnut residues in cookies, cereals, ice cream and chocolate.

Principle of the Test

The **Eurofins Immunolab Walnut** quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against walnut proteins is bound on the surface of a microtiter plate. Walnut 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 walnut 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 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 walnut 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 found on the labels of the bottles and the outer package.

1. Microtiter plate consisting of 12/6 strips with 8 bre-akable wells each, coated with anti-walnut antibodies.

2. Walnut Standards (0; 2; 6; 20; 60 ppm of walnut): 5 vials with 2.0 mL each, dyed red, ready-to-use.

3. Conjugate (anti-walnut-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. Walnut proteins adhere very strongly 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 all kinds of 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 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.

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-walnut-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 ppm 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 concentration of walnut in ppm from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

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 60 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.

Walnut (ppm)	% binding of 60 ppm
60	100
20	80
6	55
2	32
0	12

Performance

Sensitivity

The limit of detection (LOD) of the **Eurofins Immunolab Walnut test** is 0.4 ppm.

The limit of quantification (LOQ) of the **Eurofins Immunolab Walnut test** is 2 ppm.

Due to the variety of sample matrices and their influence on the blank, results less than the LOQ should be treated as negative.

Cross-reactivity

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

Adzuki	Crab, cooked	Pea
Almond	Crab, raw	Peach
Apricot	Cress	Peanut
Barley	Cumin	Pepper
Bean, white	Duck	Pine seed
Beef	Egg	Pistachio
Bovine gelatin	Ewe's milk	Plum
Brazil nut	Fenugreek	Poppy seed
Buckwheat	Fish gelatin	Pork
Caraway	Gliadin	Potato
Carob gum	Goat's milk	Prawn
Carrot	Guar gum	Pumpkin seed
Cashew	Isinglass	Rice
Cayenne	Kidney bean	Rye
Celery	Kiwi	Saccharose
Cherry	Lamb	Sesame
Chestnut	Lentil	Shrimps
Chia	Linseed	Soy
Chickpea	Lupin	Soy lecithin
Chili	Macadamia	Split peas
Cocoa	Mustard	Sunflower seeds
Coconut	Nutmeg	Tomato
Cod	Oats	Turkey
Corn	Onion	Wheat
Cow's milk	Paprika	

The following cross reactions were determined:

Hazelnut	0.0006%
Pecan	3.5%

Precision

Intra-assay Precision	4 - 9%
Inter-assay Precision	12%

Linearity

The serial dilution of spiked samples (cookies, cereals, ice cream and chocolate) resulted in a dilution linearity of 93-117%.

Recovery

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

Cookies	103%
Cereals	106%
Ice cream	87%
Chocolate	60%

References

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4. Goetz DW, et al. (2005) – Cross-reactivity among edible nuts: double immunodiffusion, crossed immunoelectrophoresis and human specific IgE serologic surveys. Ann All Asthma Immunol, 95(1):45-52
5. Roux KH, et al. (2003) – Tree nut allergens. Int Arch Allergy Immunol, 131(4):234-44