INSTRUCTION FOR USE

SENSI*Spec* ELISA Walnut 96/48 Tests

(Cat. nr. HU0030024 / HU0030048)

Enzyme Immunoassay for the Quantitative Determination of Walnut in Food

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

1. 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 Jug r 1 and Jug 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 food-stuffs are required.

The **SENSI*Spec* ELISA Walnut** represents a highly sensitive detection system and is particularly cap-able of the quantification of walnut residues in cookies, cereals, ice cream and chocolate.

2. PRINCIPLE OF THE TEST

The **SENSI*Spec* ELISA 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.

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 found 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-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.

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. 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 solid 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.

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-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.

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 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.

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 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

11. PERFORMANCE

11.1 Sensitivity

The limit of detection (LOD) of the **SENSI*Spec* ELISA Walnut test** is 0.4 ppm.

The limit of quantification (LOQ) of the **SENSI*Spec* ELISA Walnut test** is 2 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 Cross-reactivity

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

Adzuki bean	Crab, raw	Onion
Almond	Cumin	Paprika
Apricot	Date	Pea
Barley	Dill	Peach
Bean, white	Duck	Peanut
Beef	Egg, dried	Pepper, black
Beef, cooked	Ewe's milk	Pine seed
Bovine gelatin	Fennel	Plum
Brazil nut	Fenugreek	Poppy
Buckwheat	Fish gelatin	Pork
Cabbage, white	Flaxseed	Potato
Caraway seeds	Garden cress	Prawn cooked
Cardamom	Garlic, fresh	Prawn, raw
Carob gum	Garlic, granulated	Pumpkin seed
Carrot	Ginger, ground	Radish
Cashew	Ginger, fresh	Rapeseed
Cayenne	Gliadin	Rice
Celery	Goat's milk	Rye
Cherry	Guar gum	Saccharose
Chestnut	Gum arabic	Sesame
Chia seeds	Horseradish	Shrimps
Chicken	Isinglass	Soy flour
Chickpea	Kidney bean	Soy lecithin
Chili	Kiwi	Split pea
Cinnamon	Lamb	Sunflower seed
Clove	Leek	Thyme
Cocoa	Lentil	Tofu
Coconut	Lupin	Tomato
Cod	Macadamia	Turkey
Corn	Mustard,	Turmeric

	yellow	
Cow's milk	Nutmeg	Wheat
Crab, cooked	Oats	

For the following commodities of the table above the results were between 0.5*LOQ and LOQ of the kit. So, it cannot be completely excluded that these matrices may provide values above the LOQ in specific cases:

Almond	Buckwheat	Cocoa

The following cross reactions were determined:

Hazelnut	0.0004%
Pecan	3.5%
Pistachio	0.0002%

11.3 Precision

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

11.4 Linearity

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

11.5 Recovery

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

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

12. REFERENCES

1) Doi H, et al. (2008) – Reliable enzyme-linked immunosorbent assay for the determination of walnut proteins in processed foods. J Agric Food Chem, 56(17):7625-30
2) Wallowitz M, et al. (2006) – Jug r 4, a legumin group food allergen from walnut (Juglans regia Cv. Chandler). J Agric Food Chem, 54(21):8369-15
3) Niemann L, et al. (2009) – Detection of walnut residues in foods using an enzyme-linked immunosorbent assay. J Food Sci, Published Online 10 Jun 2009
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

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 results.

Gold Standard Diagnostics Budapest shall not be liable for the unsafe use of the kit out of the current European safety regulations.