INSTRUCTION FOR USE

SENSI*Spec* ELISA

Macadamia nut 96/48 Tests

(Cat. nr. HU0030013 / HU0030037)

Enzyme Immunoassay for the Quantitative Determination of Macadamia nut in Food

Sensitivity (Macadamia nut)	0.1 ppm
Recovery	83-106%
Incubation Time	60 min

1. GENERAL INFORMATION

The Macadamia tree (Macadamia integrifolia, Macadamia tetraphylla) belongs to the family of Proteaceae. With about 7.9% the fraction of proteins in macadamia nut is relatively low compared to other nuts. Some of these proteins are known for being allergenic. Compared to other nuts these proteins are still slightly characterized. Macadamia nut allergies are relatively seldom but can be very distinct in particular cases.

For macadamia nut allergic persons hidden macadamia nut allergens in food are a critical problem. Already very low amounts of macadamia nut can cause allergic reactions, which may lead to anaphylactic shock in severe cases. Because of this, macadamia nut-allergic persons should strictly avoid the consumption of macadamia nut containing food. Cross-contamination, mostly in consequence of the production process, is often noticed. This explains why in many cases the existence of macadamia nut residues in food cannot be excluded. For this reason sensitive detection systems for macadamia nut residues in foodstuff are required.

The **SENSI*Spec* ELISA Macadamia nut ELISA** represents a highly sensitive detection system for macadamia nut and is particularly capable of the quantification of residues in cookies, cereals, ice-cream, chocolate and sausage.

2. PRINCIPLE OF THE TEST

The **SENSI*Spec* ELISA Macadamia nut** quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against macadamia nut proteins is bound on the surface of a microtiter plate. Macadamia nut 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 macadamia nut 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 terminated by the addition of a stop solution, and the colour turns yellow. The yellow colour is measured photometrically at 450 nm. The concentration of macadamia nut 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 printed 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-macadamia antibodies.
2) Macadamia nut Standards (0, 1, 4, 10, 40 ppm of macadamia nut): 5 vials with 2.0 mL each, dyed red, ready-to-use.
3) Conjugate (anti-macadamia nut-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
- Extraction additive for polyphenol containing samples (HU0030100)

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. Macadamia nut proteins could adhere 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 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 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.
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 absorbances.

5) Pipet 100 µL of conjugate (anti-macadamia nut-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 (OD) value for each sample, determine the corresponding concentration of macadamia nut in ppm from the standard curve. Depending on experience and/or the availability of computer software, 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 OD% is calculated as percent of the absorption of the 40 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.

Macadamia nut (ppm)	OD% of 40 ppm
40	100
10	52
4	29
1	10
0	4

11. PERFORMANCE

11.1 Sensitivity

The limit of detection (LOD) of the **SENSI*Spec* ELISA Macadamia nut test** is 0.1 ppm for the standard curve.

Validation experiments with common matrices resulted in the following LODs [ppm].

Cookies	0.2
Cornflakes	0.1
Ice-cream	0.2
Chocolate	0.4
Sausage	0.1

The limit of quantification (LOQ) of the **SENSI*Spec* ELISA Macadamia nut test** is 1 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 Precision

Intra-assay Precision	7%
Inter-assay Precision	3-6%

11.3 Linearity

The serial dilution of spiked samples (cookies, cereals, ice-cream, chocolate and sausage) resulted in a dilution linearity of 102-112%.

11.4 Cross-reactivity

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

Adzuki bean	Crab, cooked	Paprika
Almond	Crab, raw	Pea
Apricot	Cumin	Peach
Barley	Dill	Peanut
Bean, white	Duck	Pecan
Beef, cooked	Egg	Pepper, black
Beef, raw	Eggwhite powder	Pine seed
Bovine gelatin	Ewe's milk	Pistachio
Brazil nut	Fennel	Plum
Buckwheat	Fenugreek	Poppy seed
Cabbage, white	Fish gelatin	Pork
Caraway seeds	Garden cress	Potato
Cardamom	Garlic, fresh	Prawn cooked
Carob gum	Garlic, granulated	Prawn, raw

Carrot	Ginger, ground	Pumpkin seed
Cashew	Ginger, fresh	Radish
Cayenne	Gliadin	Rapeseed
Celery	Goat's milk	Rice
Cherry	Guar gum	Rye
Chervil	Gum arabic	Saccharose
Chestnut	Horseradish	Sesame
Chia	Isinglass	Shrimps
Chicken	Kidney bean	Soy flour
Chickpea	Kiwi	Soy lecithin
Chili	Lamb	Split pea
Cinnamon	Leek	Sunflower seed
Clove	Lentil	Thyme
Cocoa	Lupin	Tofu
Coconut	Mustard, yellow	Tomato
Cod	Nutmeg	Turkey
Corn	Oats	Turmeric
Cow's milk	Onion	Wheat

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:

Chia	Chili

The following cross reactions were determined:

Flaxseed	0.0002%
Hazelnut	0.0001%
Walnut	0.0005%

11.5 Recovery

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

Cookies	106%
Cornflakes	104%
Ice-cream	92%
Chocolate	83%
Sausage	101%

12. 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.