INSTRUCTION FOR USE

SENSI*Spec* ELISA Hazelnut 96/48 Tests

(Cat. nr. HU0030010/ HU0030034)

Enzyme Immunoassay for the Quantitative Determination of Hazelnut in Food

Sensitivity (Hazelnut)	0.01-0.11 ppm
Recovery	72-105%
Incubation Time	60 min

1. GENERAL INFORMATION

Hazelnut (*Corylus avellana*) belongs to the birch plants. With 13 % the fraction of proteins in hazel-nuts is high. Many of these proteins are known for being allergenic, such as Cor a 9 and Cor a 11. Most of them are very heat resistant making them stable to different production processes. For this reason, hazelnut represents an important food allergen. For hazelnut allergic persons hidden hazelnut allergens in food are a critical problem. Already very low amounts of hazelnuts can cause allergic reactions, which may lead to anaphylactic shock in severe cases. Because of this, hazelnut allergic persons must strictly avoid the consumption of hazelnuts or hazelnut 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 hazelnut residues in foods cannot be excluded. For this reason, sensitive detection systems for hazelnut residues in foodstuffs are required.

The **SENSI*Spec* ELISA Hazelnut** represents a highly sensitive detection system and is particularly capable of the quantification of hazelnut residues in cookies, cereals, ice cream, sweets, food supplements and chocolate. Furthermore, it is validated for rinse water / CIP s and swab samples.

2. PRINCIPLE OF THE TEST

The **SENSI*Spec* ELISA Hazelnut** quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against hazelnut proteins is bound on the surface of a microtiter plate. Hazelnut 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 hazelnut 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 hazelnut 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-hazelnut antibodies.
2) Hazelnut Standards (0; 1; 4; 10; 40 ppm of hazelnut): 5 vials with 2.0 mL each, dyed red, ready-to-use
3) Conjugate (anti-hazelnut-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
- Polyvinylpyrrolidone (PVP), optional

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. Hazelnut proteins adhere very strongly to different surfaces. In certain cases they can resist a common dishwasher cleaning. 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 solid food 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 food 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 pre-heated 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.

The following variation should be applied for polyphenol containing food samples like chocolate:

Dilute 1 g of Polyvinylpyrrolidone (PVP) in 100 mL of **pre-diluted** extraction buffer. Apply the buffer as extraction buffer in the sample preparations stated above

Attention:

Do not shake the final extract to prevent from re-suspension.

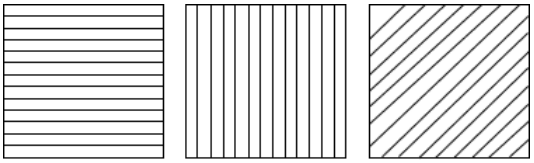
If after centrifugation a third layer at the top appears due to a high fatty matrix, only the middle aqueous phase should be applied to the wells.

The following sample preparation should be applied for rinse water samples:

1) Adjust the pH of the sample to 8.2 (+/- 0.5)
2) 1 mL of liquid sample is diluted in 4 mL of **pre-diluted** extraction and sample dilution buffer. The process is continued at point 4 of solid food sample extraction process.

The following sample preparation should be applied for swab samples on dry surfaces:

1) Mark out 5x5 cm area or use swab directly on (e.g. uneven) area.
2) Moisten the swab in 1 mL **pre-diluted** extraction and sample dilution buffer previously applied in a test tube.
3) Swab marked area by using crosshatch (1. horizontally, 2. vertically, 3. diagonally) technique while rotating the tip.



4) Place swab into the test tube.
5) Shake the test tube for 1 minute to release the sample from the swab. The process is continued at point 4 of solid food sample extraction process.

For wet surfaces exactly the same procedure is applied without prior need to moisten the swab.

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-hazelnut-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 following evaluation procedure should be applied for all **food samples** prepared by the procedure as stated *Sample Preparation*:

The ready-to-use standards are prepared for a direct determination of food sample concentrations. The dilution (1:20) 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 hazelnut in ppm from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

The following evaluation procedure should be applied for **rinse water samples** prepared according the procedure stated *Sample Preparation*:

1) Apply the evaluation procedure food samples as stated above.
2) Divide the result by 4 in order to compensate the different dilution factor of the extraction procedure to receive the sample concentration in mg/L.

The following evaluation procedure should be applied for **swab samples** prepared according the procedure stated in *Sample Preparation*:

1) Apply the evaluation procedure food samples as stated above.
2) Multiply the result (ppm) by 2 in order to compensate the different dilution factor of the extraction procedure to receive the sample concentration in ng/cm^2.

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 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 every new test.

Hazelnut (ppm)	% binding of 40 ppm
40	100
10	59
4	38
1	11
0	2

11. PERFORMANCE

11.1 Sensitivity

The limit of detection (LOD) of the **SENSI*Spec* ELISA Hazelnut test** is 0.07 ppm hazelnut.
Validation experiments with common matrices resulted in the following mean LODs [ppm].

Cookies	0.11
Ice-cream	0.05
Sweets	0.02
Chocolate	0.01
Food Supplement	0.04

The limit of quantification (LOQ) of the **SENSI*Spec* ELISA Hazelnut test** is 1 ppm hazelnut.
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	4.9%
Inter-Assay Precision	2.9%
Inter-Extraction Precision	5.3%

11.3 Linearity

The serial dilution of spiked samples (cookies, cereals, ice cream, chocolate, sweets and food supplement) resulted in a mean dilution linearity of 91-113%.

11.4 Cross-reactivity

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

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

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:

Bean, white	Cinnamon	Mustard
Brazil nut	Flaxseed	Poppy seed
Cashew	Ginger, fresh	Sesame
Cayenne	Kidney bean	Turmeric
Chili	Lentil	

The following cross reactions were determined:

Almond	0.0001%
Cocoa	0.0001%
Clove	0.0003%
Coconut	0.0001%
Pecan	0.0012%
Walnut	0.0024%

11.5 Recovery

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

Cookies	102%
Cereals	92%
Ice cream	82%
Chocolate	83%
Sweets	83%
Food Supplement	77%

12. LIABILITY

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