INSTRUCTION FOR USE

SENSI*Spec* ELISA Lupin 96/48 Tests

(Cat. nr. HU0030011/ HU0030035)

Enzyme Immunoassay for the Quantitative Determination of Lupin in Food

Sensitivity (Lupin)	0.2 ppm
Recovery	98-113%
Incubation Time	60 min

1. GENERAL INFORMATION

Lupin belongs to the legume family. With about 35-45% the fraction of proteins in lupin seed is very high. Some of these proteins, predominantly those in the α-, β- and γ-Conglutin fractions are known for being allergenic, due to cross-reaction to allergens of soy and peanut. Because of its convenient nutritional properties the use of lupin flour as a food ingredient is highly increasing. Lupin is often used in dietary products, especially for milk allergic people, vegetarians and patients with celiac disease. In food production three (sweet) lupin species are commonly used: Lupinus albus, Lupinus angustifolius and Lupinus luteus.

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

The **SENSI*Spec* ELISA Lupin** represents a highly sensitive detection system for lupin and is particularly capable of the quantification of residues in sausage, bakery products, potato products, ketchup and juices. Due to high cross-reactivity the test is suitable for the detection of all commonly used lupin species.

2. PRINCIPLE OF THE TEST

The **SENSI*Spec* ELISA Lupin** quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against lupin proteins is bound on the surface of a microtiter plate. Lupin 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 lupin 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 lupin 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-lupin antibodies.
2) Lupin Standards (0; 2; 5; 15; 30 ppm of lupin): 5 vials with 2.0 mL each, dyed red, ready-to-use.
3) Conjugate (anti-lupin-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. Lupin proteins could adhere to different surfaces. To identify possible cross-contamination caused by previous extractions it is strongly recom-mended 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) Due to high matrix effects samples containing more than 50% pure beef should be further diluted 1 + 1 with **pre-diluted** extraction and sample dilution buffer.
5) 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-lupin-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 lupin 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 30 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.

Lupin (ppm)	% binding of 30 ppm
30	100
15	75
5	38
2	21
0	5

11. PERFORMANCE

11.1 Sensitivity

The limit of detection (LOD) of the **SENSI*Spec* ELISA Lupin test** is 0.2 ppm for the standard curve.

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

Sausage	0.2
Bread	0.3
Orange juice	0.7
Ketchup	0.1
Croquette	0.2

The limit of quantification (LOQ) of the **SENSI*Spec* ELISA Lupin 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 Precision

Intra-assay Precision	6-9%
Inter-assay Precision	4-9%

11.3 Linearity

The serial dilution of spiked samples (sausage, bread, orange juice, ketchup and croquette) resulted in a dilution linearity of 93% - 107%.

11.4 Cross-reactivity

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

Apricot	Dill	Peanut
Almond	Duck	Pepper, black
Barley	Egg	Pine seed
Bean, white	Fennel	Pistachio
Beef, cooked	Flaxseed	Poppy
Beef, raw	Garden cress	Pork

Bovine gelatin	Garlic, fresh	Potato
Brazil nut	Garlic, granulated	Prawn, cooked
Buckwheat	Ginger, fresh	Prawn, raw
Cabbage, white	Ginger, ground	Pumpkin seed
Caraway	Gliadin	Radish
Cardamom	Goat's milk	Rapeseed
Carob gum	Guar gum	Rice
Carrot	Gum arabic	Rye
Cashew	Hazelnut	Saccharose
Cayenne	Horseradish	Sesame
Celery	Kiwi	Shrimps
Cherry	Lamb	Split pea
Chestnut	Leek	Strawberry
Chia	Macadamia	Sunflower seed
Chicken	Mustard	Thyme
Chili	Oats	Tofu
Coconut	Onion	Tomato
Cod	Orange	Turkey
Corn	Paprika	Turmeric
Cow's milk	Pea	Walnut
Cumin	Peach	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:

Bean, white	Ginger, ground	Split pea
Cayenne	Guar gum	Thyme
Cherry	Hazelnut	

The following cross reactions were determined:

Adzuki bean	0.0003%
Chickpea	0.0003%
Cinamon	0.0005%
Clove	0.0009%
Cocoa	0.0003%
Fenugreek	0.0006%
Kidney bean	0.0003%
Lentil	0.0003%
Nutmeg	0.0003%
Pecan	0.0003%
Plum	0.0003%
Soy flour	0.0065%
Soy lecithin	0.0003%

11.5 Recovery

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

Sausage	99%
Bread	113%
Orange juice	104%
Ketchup	98%
Croquette	111%

Note: *The industrial process of food transformation has an impact on proteins which might affect testing performance. For this reason, recovery/cross reactivity may be altered for heat treated samples.*

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 the results.

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