INSTRUCTION FOR USE

SENSI*Spec* ELISA Ovalbumin 96/48 Tests

(Cat. nr. HU0030017 / HU0030041)

Enzyme Immunoassay for the Quantitative Determination of Ovalbumin in Food

Sensitivity (Ovalbumin)	4 ppb
Recovery	95 - 115%
Incubation Time	60 min

1. GENERAL INFORMATION

Hen's egg (Gallus gallus) is very rich of proteins and represents an important food source for humans. While proteins of egg yolk only have minor allergenicity, many proteins of egg white are known to be allergenic. In addition to lysozyme, ovomucoid ovotransferrin and livetin, ovalbumin represents the main fraction of the egg white allergens. Amongst others egg powder as well as pure ovalbumin is often used as fining reagent for wine. For allergic persons the consumption of ovalbumin represents a critical problem. Already very low amounts of the allergen can cause allergic reactions, which may lead to anaphylactic shock in severe cases. Because of this, ovalbumin allergic persons must strictly avoid the consumption of ovalbumin containing food. Non-declared addition of ovalbumin in food is hazardous for allergic people. Cross-contamination, mostly in consequence of the production process, is often noticed. Since July 1, 2012 the European Union requests allergen labeling for wine if milk or egg proteins are used during the production and are still present at a detection level of 0.25 mg/L or greater. Thus for the detection of ovalbumin residues, sensitive assay systems are required.

The **SENSI*Spec* ELISA Ovalbumin** represents a highly sensitive detection system for ovalbumin and is particularly capable of the quantification of ovalbumin residues in wine.

2. PRINCIPLE OF THE TEST

The **SENSI*Spec* ELISA Ovalbumin** quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against ovalbumin is bound on the surface of a microtiter plate. Ovalbumin 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 ovalbumin 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 ovalbumin 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-ovalbumin antibodies.
2) Ovalbumin standards (0; 25; 100; 250; 500 ppb of ovalbumin): 5 vials with 2.0 mL each, dyed red, ready-to-use.
3) Conjugate (anti-ovalbumin-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. 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 wine samples:

1) 1 ml of the wine sample is diluted in 19 mL of **pre-diluted** extraction buffer.
2) 100 µL of the wine 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 all other 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.

Attention:

- *Do not shake the final extract to prevent from re-suspension.*
- *If after centrifugation a third layer at the top ap-pears 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 re-commended.
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-ovalbumin-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 ppb 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 ovalbumin in ppb 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 500 ppb 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.

Ovalbumin (ppb)	% binding of 500 ppb
500	100
250	55
100	26
25	9
0	1

11. PERFORMANCE

11.1 Sensitivity

The limit of detection (LOD) of the **SENSI*Spec* ELISA Ovalbumin test** is 4 ppb.

The limit of quantification (LOQ) of the **SENSI*Spec* ELISA Ovalbumin test** is 25 ppb.

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	Dill	Peach
Almond	Duck	Peanut
Apricot	Fennel	Pecan
Barley	Fenugreek	Pepper, black
Bean, white	Fish gelatin	Pine seed
Beef, cooked	Flaxseed	Pistachio
Beef, raw	Garden cress	Poppy
Bovine gelatin	Garlic, fresh	Pork
Brazil nut	Garlic, granulated	Potato
Buckwheat	Ginger, ground	Prawn cooked
Cabbage, white	Ginger, fresh	Prawn, raw
Caraway seeds	Gliadin	Pumpkin seed
Cardamom	Goat's milk	Radish
Carob gum	Guar gum	Rapeseed
Carrot	Gum arabic	Rice
Cashew	Hazelnut	Rye
Cayenne	Horseradish	Saccharose
Celery	Isinglass	Sesame
Cherry	Kidney bean	Sheep`s milk
Chestnut	Kiwi	Shrimps
Chia	Lamb	Soy flour
Chicken	Leek	Soy lecithin
Chickpea	Lentil	Split pea
Chili	Lupin	Sunflower seed
Cinnamon	Macadamia	Thyme
Clove	Mustard, yellow	Tofu
Cocoa	Non Fat dry	Tomato

	milk	
Coconut	Nutmeg	Turkey
Cod	Oats	Turmeric
Corn	Onion	Walnut
Cow' milk	Paprika	Wheat
Cumin	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:

Fish gelatin	Split pea	Turkey
Gum arabic		

The following cross-reactions were determined:

Conalbumin	< 0.2%
Whole Egg Powder	15%
Lysozyme	< 0.02%
Ovomucoid	< 0.02%

11.3 Precision

Intra-assay Precision	3 - 4%
Inter-assay Precision	2 – 3%

11.4 Linearity

The serial dilution of spiked samples (white wine, red wine) resulted in a dilution linearity of 94% - 96%.

11.5 Recovery

By spiking wine samples (white wine, rosé wine, red wine) with different amounts of ovalbumin, recoveries resulted in 95-115%.

12. REFERENCES

1) Rolland JM, et al. (2008) – Specific and sensitive enzyme-linked immunosorbent assays for analysis of residual allergenic food proteins in commercial bottled wine. J Agric Food Chem, 56(2):349-54
2) Kirschner S, et al. (2009) – Allergenicity of wine containing processing aids: A double-blind, placebo-controlled food challenge. J Inv Clin Imm, 19(3):210-17
3) Weber P, et al. (2007) – Investigation of the allergenic potential of wines fined with various proteinogenic fining agents by ELISA- J Agric Food Chem, 55(8):3127-33

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.