INSTRUCTION FOR USE

SENSI*Spec* ELISA Casein 96/48 Tests

(Cat. nr. HU0030003 / HU0030027)

Enzyme Immunoassay for the Quantitative Determination of bovine Casein in Food

Sensitivity (Casein)	0.03 ppm
Recovery	82-102%
Incubation Time	60 min

1. GENERAL INFORMATION

Bovine milk belongs to the most important allergenic food ingredients especially for children. Already very low amounts of bovine milk can cause allergic reactions, which may lead to anaphylactic shock in severe cases. Because of this, milk allergic persons must strictly avoid the consumption of milk or milk containing food. In particular the presence of hidden milk proteins such as in sausage, cookies, convenience food or beverages represent a critical problem for milk allergic persons. According to EU Directive 2003/89/EG the addition of bovine milk has to be labeled. For the detection of bovine milk in foodstuffs, sensitive detection systems are required.

Approximately 80% of bovine milk proteins are caseins which are composed of α-, β- and κ-caseins. So, these heat-stable allergens represent the main fraction of bovine milk proteins.

The **SENSI*Spec* ELISA Casein** represents a highly sensitive detection system and is particularly capable of the identification and quantification of bovine casein residues in cookies, bread crumbs, sausage, orange juice, wine, soy products and chocolate.

2. PRINCIPLE OF THE TEST

The **SENSI*Spec* ELISA Casein** quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody directed against casein is bound on the surface of a microtiter plate. Casein 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 casein 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 casein 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-casein antibodies.
2) Casein Standards (0, 0.2, 0.6, 2, 6 ppm of casein): 5 vials with 2.0 mL as 100x concentrate, dyed brownish. Dilute 20 µL of standard with 1980 µL **pre-diluted** extraction and sample dilution buffer to achieve the concentrations named above. Stored at 4°C the diluted standards are stable for at least 24 hours.

Note: The concentrations above refer to the 100x diluted standards.

3) Conjugate (anti-casein-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 (Carbonate buffer): 2/1 x 120 mL as 5x concentrate, dyed red. Dilute 1+4 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
- Analytical balance
- Mortar, mixer
- Water bath
- Centrifuge
- ELISA reader (450 nm)

6.2 Reagents

- Double distilled water

7. SAMPLE PREPARATION

Due to a 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 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) 0.5 g of the homogenized mixture is suspended in 10 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 meat and sausage samples should be further diluted 1 + 4 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:
0.5 mL of liquid sample is diluted in 9.5 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.
For wine samples the incubation for 15 min at 60°C as well as the centrifugation step are not necessary and can be omitted.

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 **diluted** standards should be determined at least twofold. When samples in great numbers 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 of **diluted** 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-casein-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 **diluted** 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 meat containing samples or 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 casein in ppm from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.

For calculation of the amount of a corresponding raw product, the casein concentration has to be multiplied with a product specific conversion factor (F).

The following conversion factors have been determined by means of validation experiments:

Whole milk	42
Skim milk powder (MoniQA MQA 092014)	4.4
Skim milk powder (NIST RM1549)	3.6
Whole Milk Powder (NIST RM8435)	4.9
Caseinate	1.2

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 6 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.

Casein (ppm)	% binding of 6 ppm
6	100
2	75
0.6	44
0.2	23
0	8

11. PERFORMANCE

11.1 Sensitivity

Based on the values derived from blank matrix signal + 3 x SD, a general LOD for the **SENSI*Spec* ELISA Casein test** was determined as 0.03 ppm.

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

Cookies	0.02
Bread Crumbs	0.03
Sausage	0.02
Chocolate	0.00
White wine	0.02
Red Wine	0.02
Soy Milk	0.02
Orange Juice	0.03

The limit of quantification (LOQ) of the **SENSI*Spec* ELISA Casein test** is 0.2 ppm casein.

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 Specificity

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

Adzuki bean	Egg	Peach
Almond	Fennel	Peanut
Barley	Fenugreek	Pecan
Bean, white	Flaxseed	Pepper, black
Beef	Garden cress	Pineseed
Beta-lactoglobulin	Garlic, fresh	Pistachio
Bovine gelatin	Garlic, granulated	Poppy seed
Brazil nut	Ginger, dried	Pork

Buckwheat	Ginger, fresh	Potato
Cabbage, white	Gliadin	Prawn
Caraway	Guar gum	Pumpkin seed
Cardamom	Gum arabic	Radish
Carrot	Hazelnut	Rapeseed
Cashew	Hempseed	Rice, brown
Celery	Horseradish	Rice, white
Cherry	Kidney bean	Rye
Chestnut	Kiwi	Saccharose
Chia	Lamb	Shrimps
Chicken	Leek	Soy flour
Chickpea	Lentil	Soy lecithin
Chili	Lima bean	Split pea
Cinnamon	Lupin	Sunflower seed
Clove	Macadamia	Thyme
Cocoa	Mustard	Tofu
Coconut	Nutmeg	Tomato
Corn	Oats	Turkey
Cumin	Onion	Turmeric
Dill	Paprika	Walnut
Duck	Pea	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:

Beef	Lamb	Pork
Prawn	Rapeseed	

The following cross-reactions were determined:

Cod	0.00002%
Ewe's milk	< 1.2%
Goat's milk	< 1.1%
Sesame	0.00004%

11.3 Precision

Intra-assay Precision	5 - 11%
Inter-assay Precision	8 - 14%

11.4 Linearity

The serial dilution of spiked samples (cookies, bread crumps, chocolate, sausage, soy milk, orange juice and white wine) resulted in a dilution linearity of 100% - 127%.

11.5 Recovery

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

Cookies	99%
Bread crumps	84%
Chocolate	85%
Sausage	82%
Soy milk	89%
Orange juice	87%
White wine	102%
Red wine	102%

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.