

INSTRUCTION FOR USE

SENSISpec ELISA Milk

96/48 Tests

(Cat. nr. HU0030014 / HU0030038)



Enzyme Immunoassay for the Quantitative Determination of bovine milk protein in Food

Sensitivity (milk protein)	0.01-0.24 ppm
Recovery	91-109%
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. β -Lactoglobulin, the major allergen of whey, represents further 10% of the total protein

The **SENSISpec ELISA Milk ELISA** represents a highly sensitive detection system for milk proteins based on NIST 1549a reference material. The test is likewise capable of the quantification of casein and β -lactoglobulin residues in food and is validated for almond milk, soy milk, oat milk, coconut milk, cookies, sausages and meat, chocolate, orange juice, spices, sweets and wine.

2. PRINCIPLE OF THE TEST

The **SENSISpec ELISA Milk** quantitative test is based on the principle of the enzyme linked immunosorbent assay. An antibody mixture is bound on the surface of a microtiter plate. Milk protein 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 mixture directed against milk 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 milk proteins 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 milk protein binding antibodies.

- 2) Milk protein Standards, based on NIST RM 1549a reference material: 5 vials with 2.0 mL (0, 0.4, 1, 4, 10 ppm of milk protein), 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-milk protein-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 H₂SO₄): 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

- 10, 100 - 1000 µL micropipets
- Analytical balance
- Mortar, mixer
- Water bath
- Centrifuge
- ELISA reader (450 nm)

6.2. Reagents

- Double distilled water
- Extraction additive (HU0030100), optional, can be ordered separately

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. Milk proteins could 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 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) 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 pre-heated 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 or higher. 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:

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.

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

Add 1 g of extraction additive to 0.5 g/0.5 mL of sample before adding the **pre-diluted** extraction and sample dilution buffer and continue the extraction process as stated above.

Attention:

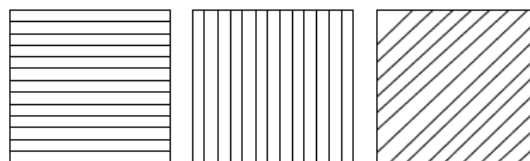
- *Do not shake the final extract to prevent from re-suspension.*
- *If after centrifugation a third layer at the top appears due to a high fatty matrix, only the middle aqueous phase should be applied to the wells.*

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

- 1) Adjust the pH of the sample to 9.5 (+/- 0.2)
- 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 **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-milk protein-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 H₂SO₄) 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 pre-diluted 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 milk 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/cm².

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

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

Non fat milk powder (NIST RM1549)	3.6
Non fat milk powder (MoniQA 092014)	3.3
Whole milk powder (NIST RM1549a)	4.8
Total milk	33
Yoghurt	31
Curd	19
Caseinate	1.0
Beta lactoglobulin	1.0

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 10 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.

Milk protein (ppm) % binding of 10 ppm

10	100
4	71
1	33

0.4	21
0	10

11. PERFORMANCE

11.1 Sensitivity

The limit of detection (LOD) of the **SENSISpec ELISA Milk** test is 0.21 ppm of milk protein related to the standard curve.

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

Almond milk	0.08
Soy milk	0.12
Oat milk	0.12
Coconut milk	0.04
Cookies	0.13
Sausage / meat	0.18
Chocolate	0.05
Orange juice	0.09
Spices	0.13
Sweets	0.09
Wine	0.07

The limit of quantification (LOQ) of the **SENSISpec ELISA Milk** test is 0.4 ppm of milk protein.

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	5.7%
Inter-Extraction Precision	6.5%

11.3 Linearity

The serial dilution of spiked samples (almond milk, soy milk, oat milk, coconut milk, sausage / meat, chocolate, orange juice, spices, sweets and wine) resulted in a dilution linearity of 92- 108%.

11.4 Specificity

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

Adzuki bean	Dill	Peach
Almond	Duck	Peanut
Apricot	Egg, dried	Pecan
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	Gladiolus	Radish
Carob gum	Guar gum	Rapeseed
Carrot	Gum arabic	Rice
Cashew	Hazelnut	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
	Mustard,	Tofu
	yellow	
Cinamon	Nutmeg	Tomato
Clove	Oats	Turkey
Cocoa	Onion	Turmeric
Coconut	Paprika	Walnut
Cod	Pea	Wheat
Corn		
Cumin		

The following cross-reactions were determined:

Ewe's milk	0.25%
Goat's milk	0.30%

11.5 Recovery

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

Almond milk	101%
Soy milk	94%
Oat milk	99%
Coconut milk	107%
Cookies	99%
Sausage / meat	101%
Chocolate	100%
Orange juice	100%
Spices	92%
Sweets	102%
Wine	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 the results.

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