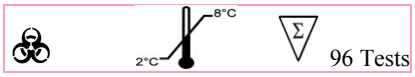
Diagnostic Automation / Cortez Diagnostics, Inc.
IMMUNO DIAGNOSTICS

AccuDiag™ Milk ELISA

REF 5165-8



Test	Milk ELISA
Recovery	79 - 122%
Incubation Time	60 min
Sensitivity (Macadamia nut)	0.05 ppm

INTENDED USE

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. ß-Lactoglobulin, the major allergen of whey, represents further 10% of the total protein

The DAI Milk ELISA represents a highly sensitive detection system for milk proteins based on NIST 1549 reference material. The test is likewise capable of the quantification of casein and ß-lactoglobulin residues in food and is validated for cookies, bread crumbs, sausage, orange juice, wine, soy products and chocolate.

TEST PRINCIPLE

The DAI Milk ELISA test is based on the principle of the enzyme linked immunosorbent as-say. 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 in-cubated 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 photo-metrically at 450 nm. The concentration of milk proteins is directly proportional to the colour intensity of the test sample.

MATERIALS AND COMPONENTS

Materials provided with the test kits

The kit contains reagents for 96 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 strips with 8 breakable wells each, coated with milk protein binding antibodies.
2. Milk protein Standards, based on NIST RM 1549 reference material: 5 vials with 4.0 mL (0, 0.4, 1, 4, 10 ppm of milk protein), as 100x concentrate, dyed blue. 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 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 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.
9. Instruction Manual.

Materials required but not provided

Instrumentation

1. 100 - 1000 µL micropipettes
2. Analytical balance
3. Mortar, mixer
4. Water bath
5. Centrifuge
6. ELISA reader (450 nm)

Reagents

Double-distilled water

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 representative-ness 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 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.

PRECAUTION

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 (micropipettes, ELISA reader etc.).

HEALTH & 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).

ASSAY 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 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-macadamia nut-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 color will turn yellow upon addition.
11. After thorough mixing, measure absorbance at 450 nm (reference wavelength 620 nm), using an ELISA reader. The color is stable for 30 minutes.

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 macadamia nut 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 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:

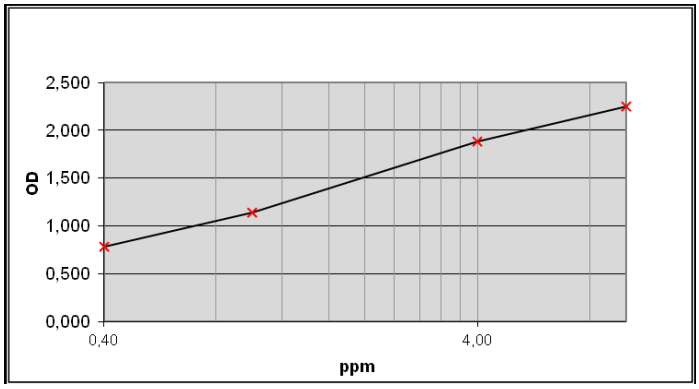
Non fat milk powder (NIST RM1549)	2.7
Whole milk powder (NIST RM8435)	4.4
Caseinate	1.0
ß-Lactoglobulin	1.1

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

Milk protein (ppm)	% binding of 10 ppm
10	100

6	86
1	50
0.4	32
0	4



Precision

Intra-assay Precision	8-10%
Inter-assay Precision	10-17%

Linearity
The serial dilution of spiked samples (cookies, bread crumbs, chocolate, sausage, soy milk, orange juice and white wine) resulted in a dilution linearity of 80 - 130%.

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

Cookies	102%
Bread crumbs	110%
Chocolate	99%
Sausage	88%
Soy milk	79%
Orange juice	106%
White wine	122%

PERFORMANCE CHARACTERISTICS

Sensitivity
The limit of detection (LOD) of the **DAI Milk ELISA** test is 0.05 ppm of milk protein.

Validation experiments with common matrices resulted in the following LODs (ppm).

Soy milk	0.13
Orange juice	1.10
White wine	0.03
Bread crumbs	0.08
Cookies	0.16
Chocolate	0.10
Sausage	0.18

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

Due to the variety of sample matrices and their influence on the blank, results less than the LOQ should be treated as negative.

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

Egg	Sesame	Almond
Wheat	Mustard	Cocoa
Rye	Lupin	Beef
Barley	Celery	Pork
Oats	Peanut	Chicken
Rice	Hazelnut	Cod
Corn	Pistachio	
Soy	Walnut	

The following cross reactions were determined:

Ewe's milk	0.94%
Goat's milk	0.01%

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