PLUS CHEESE FRAUD ELISA

5171BKCC[5]11.20

A competitive enzyme immunoassay for screening of the presence of bovine milk in cheese of other species and sources

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BRIEF INFORMATION

The Plus Cheese Fraud ELISA is a competitive enzyme immunoassay for screening for the presence of bovine milk in cheese produced from non bovine milk.
The ELISA-kit contains a 96 wells microtiter plate, calibration standards and all reagents to perform the assay. Samples and standards are measured in duplicate which means that a total of 41 samples can be analyzed per kit.

1. INTRODUCTION

EuroProxima provides 3 ELISA kits for the detection of adulteration of food with bovine milk. The tests are based on monoclonal antibodies directed against small epitopes specific for bovine κ -casein protein. The kits for the detection of adulteration with bovine milk (cat. code 5171BKCM) and plus bovine rennet whey (cat. code 5171BRW) in milk from other species and sources are based on the antibodies directed against the glycomacropeptide (GMP) part of bovine κ-casein. For fraud with cheese identity, a monoclonal antibody directed against a small epitope on the para-κ-casein part of the protein is used. All the applied antibodies are specific for the detection of cow's and buffalo's milk. Moreover, the assays recognize denatured bovine-κ-casein in heat-treated and/or matured products due to the application of antibodies directed against only a small epitope in an inhibition assay format.

2. PRINCIPLE OF THE ELISA

The principle of the test is based on the binding of a HRP labeled monoclonal antibody (mAb) directed against an epitope on para κ-casein. κ-Casein is coated to the wells of the microtiter plate, HRP labeled antibody and sample or standard are added to the wells. The antibody competes for the binding sites of para κ-casein on the plate and free para κ-casein in the samples or standards.
The amount of mAb-HRP bound to the para κ-casein part of the κ-casein coated in the well is visualised by the addition of a chromogen substrate tetramethylbenzidine (TMB). Bound mAb-HRP transforms the colourless chromogen into a coloured product. The substrate reaction is stopped by addition of the sulphuric acid. The colour intensity is measured photometrically at 450 nm and it is inversely proportional to the concentration of para κ-casein in the sample.

3. SPECIFICITY AND SENSITIVITY

The mAb applied in the Plus Cheese Fraud ELISA recognises a small 7 amino acids peptide fragment of the para-k-casein part of bovine k-casein (cow and buffalo). This specific epitope is not present on the proteins from milk of other species (i.e. goat, sheep, camel, etc.).

Due to this small epitope being detected in competitive assay format, the Plus Cheese Fraud ELISA can be also used to screen for k-casein after proteolysis (breakdown of proteins into smaller polypeptides) in old cheeses.

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

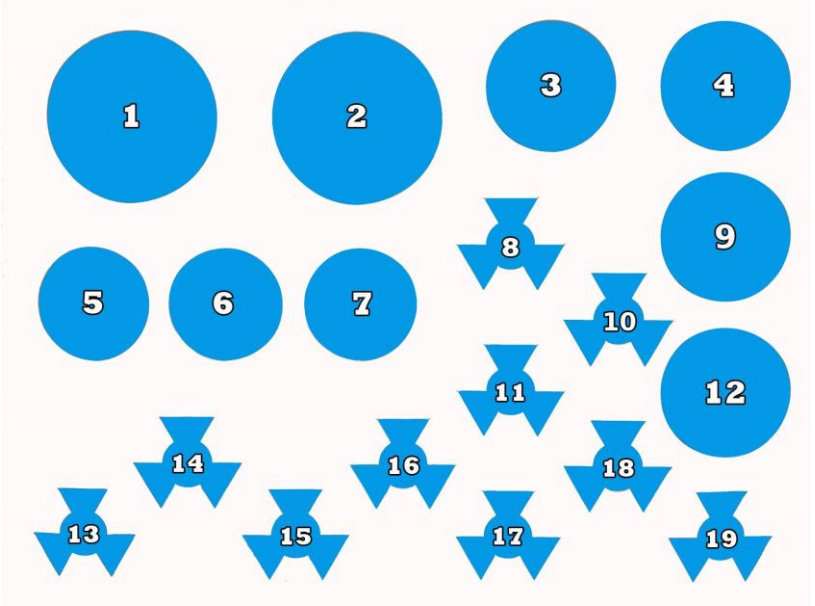
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with k-casein. Plate is ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Sample dilution buffer** (20 ml, 4x concentrated)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. **Standard** (lyophilized)
6. **Standard** (lyophilized)
7. **Standard** (lyophilized)
8. **Conjugate** (100 µl, 100x concentrated)
9. **Conjugate Dilution buffer** (15 ml ready-to-use)
10. Not in use
11. Not in use
12. Not in use
13. Not in use
14. Not in use
15. Not in use
16. Not in use
17. Not in use
18. Not in use
19. Not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 15 ml centrifuge tubes
- Homogeniser
- Vortex mixer
- Head-over-head shaker
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 10 - 100 - 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Distilled water (bidest)
- Centrifuge
- Sodium hydroxide
- Urea
- TRIS-HCl, Tris(hydroxymethyl)aminomethane hydrochloride

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

8. SAMPLE PREPARATION

Cheese samples
- Pulverize the cheese sample
- Weigh 1 g of the cheese sample in a tube and add 9 ml of 0.5 M urea buffer (see chapter 9)
- Homogenise by mixing (ultra turrax or stick blender) for 30 seconds and mix head over head for 30 minutes
- Centrifuge 10 minutes at 2000 x g
- If a fat layer is formed, remove it with a spatula
- Pipette 50 µl of the cheese extract and add 950 µl of sample dilution buffer (see chapter 9)
- Homogenise by vortexing for 2 seconds
- 50 µl of this solution is used in the ELISA test.

9. PREPARATION OF REAGENTS

Before starting the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at 2°C to 8°C.

Microtiter plate
Return unused strips into the resealable bag with desiccant and store at 2°C to 8°C for use in subsequent assays. Retain also the strip holder.

Conjugate
The conjugate is 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Make sure there remains no conjugate in the cap. Add 10 µl of the concentrated conjugate solution to 990 µl of the conjugate dilution buffer. Per 2 x 8 wells 800 µl of diluted conjugate is required.
Store unused concentrated conjugate at 2°C to 8°C.

Sample dilution buffer
The sample dilution buffer is 4x concentrated. The concentrated buffer should be brought to room temperature and thoroughly mixed before dilution (20 ml buffer + 60 ml distilled water). Concentrated buffer can show precipitates. Mix well before dilution with distilled water.

Standards
Prepare κ-casein dilution series in the sample dilution buffer. Add 1 ml of the sample dilution buffer to a lyophilised standard vial and mix. This solution contains κ-casein at the concentration of 25 µg/ml. Prepare the dilution series in a following way:

10 µg/ml	200 µl of standard 25 µg/ml	+ 300 µl of sample dilution buffer
4 µg/ml	200 µl of standard 10 µg/ml	+ 300 µl of sample dilution buffer
1.6 µg/ml	200 µl of standard 4 µg/ml	+ 300 µl of sample dilution buffer
0.64 µg/ml	200 µl of standard 1.6 µg/ml	+ 300 µl of sample dilution buffer

Prepare the standards freshly before use.

Rinsing buffer
The rinsing buffer is 20x concentrated. Prepare fresh dilution before use. For each strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at 4ºC. Make sure that this vial is at room temperature before use (keep in the dark) and mix the content before pipetting into the wells.
Store at 4°C until the expiry date stated on the kit label.

0.5 M Urea buffer
Add 3.15 g of TRIS-HCL to 900 ml distilled water.
Adjust the pH to 8.5 with NaOH.
Add 30.3 g of urea and mix well using the magnetic stirrer.
Adjust the volume to 1000 ml with distilled water.
Store at 4°C for 1 month.

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. Each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
Manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual rinsing solution from the wells.
6. Do not allow the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, make sure that all wells can be aspirated completely and that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to Chapter 8 and prepare reagents according to Chapter 9

2. Pipette 100 µl of the sample dilution buffer in duplicate (wells G1, G2, blanks).
   Pipette 50 µl of the sample dilution buffer in duplicate (wells A1, A2, maximal signal).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to F1,2 i.e. 0.64, 1.60, 4, 10 and 25 µg/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 50 µl of conjugate into all wells, except the blanks G1 and G2.

5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

6. Incubate for 1 hour in the dark at 20°C to 25°C.

7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

8. Pipette 100 µl of substrate solution into each well.

9. Incubate 30 minutes at 20°C to 25°C.

10. Pipette 100 µl of stop solution to each well.

11. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean absorbance of the wells G1 and G2 (Blank) from the individual absorbance values of the wells containing the standards and the samples.
The O.D. values of the five standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard (Bmax, wells A1 and A2) and multiplied by 100.
The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard (Bmax)}} \times 100 = \text{percentage maximal absorbance}$$

Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted on the Y-axis versus analyte equivalent concentration (µg/ml) on the logarithmic X-axis.
The concentration of k-casein present in the sample can be read from the calibration curve. There is a correlation between the concentration of bovine k-casein and the percentage of cow's milk in the sample. However, the correlation factor depends on the k-casein concentration in cow's milk. The k-casein concentration in cow's milk may vary from 2 to 4 mg/ml.
Based on the results obtained with soft and hard cheese samples containing known percentages of cow's milk, we concluded that the addition of 1% cow's milk in cheese results in an inhibition of Bmax of >50%. Samples showing a percentage of maximal absorbance >70% should be considered negative, 70-50% suspected and <50% positive.

12. LITERATURE

Haasnoot W, Sajic N, Doorn Essers K, Streppel L, Verheijen R. (2014) ELISA for Raw and Heat-Treated Cow's and Buffalo's Milk in the Milk of Other Species and Sources. Journal of Advances in Dairy Research 2: 118.

Bremer MGEG, Kemmers-Voncken AEM, Boers EAM, Frankhuizen R, Haasnoot W (2008) Enzyme-linked immunosorbent assay for the detection of bovine rennet whey powder in milk powder and buttermilk powder. International Dairy Journal 18, 294-302.

Haasnoot W, Smits NG, Kemmers-Voncken AE, Bremer MG. (2004) Fast biosensor immunoassays for the detection of cows' milk in the milk of ewes and goats. J Dairy Research 71(3):322-329.

13. ORDERING INFORMATION

For ordering the Plus Cheese Fraud ELISA kit, please use cat. code 5171BKCC.

14. REVISION HISTORY

"Plus" has been added to the already existing name of the manual. The kit and the kit ordering code will remain unchanged.

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