Plus Cow's Milk ELISA

5171MILK[1]03.20

A competitive enzyme immunoassay for the detection of raw and heat-treated cow's milk in milk from other species

EUROPROXIMA PLUS COW'S MILK ELISA

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BRIEF INFORMATION

The Plus Cow's Milk ELISA is a competitive enzyme immunoassay for the detection of raw and heat-treated cow's milk in milk of other species. With this ELISA kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 40 samples can be analysed with one kit. The ELISA kit contains all reagents required to perform the assay.

1. INTRODUCTION

The Plus Cow's Milk ELISA is a fast screening tool for the detection of cow's milk residues in milk products from other species. The test can be used for such purposes as:

- Quality control of the incoming goods, for example for the detection of residues of cow's liquid milk in goat's liquid milk batch to detect contamination before a batch is processed further.
- Quality control of a final product, for example for the detection of residues of cow's milk powder in goat's milk powder as a result of cross-contamination between production batches.

2. PRINCIPLE OF THE ELISA

The Plus Cow's Milk ELISA uses a specific mouse monoclonal antibody (mAb) raised against the bovine milk protein κ-casein which is labelled with the enzyme horseradish peroxidase (HRP). The binding of this antibody-enzyme conjugate to the ready-to-use κ-casein-coated 96-wells microtiter plate is inhibited by the κ-casein from the sample. The lyophilised κ-casein standard provided in the kit is used to prepare a calibration series. The concentration of κ-casein in the standard after reconstitution was optimised to correspond to the cow's milk concentration of 4% (v/v or w/w). There is a linear correlation between the amount of detected κ-casein and added percentages of cow's milk which was experimentally found during development of the ELISA kit. When a calibration series is prepared from the provided standard a semi-quantitation can be achieved in the range from 0.125 to 4% of added cow's milk (v/v or w/w).
Antibody-enzyme conjugate and standard/sample are mixed together in the microtiter plate well. After an incubation of 30 min, the non-bound reagents are removed in a washing step. The amount of bound antibody-enzyme conjugate is visualised by the addition of a substrate solution (H_2O_2/TMB). Bound conjugate transforms the colourless chromogen into a coloured product. The substrate reaction is stopped by the addition of sulphuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the concentration of cow's milk in the sample.

3. SPECIFICITY AND SENSITIVITY

Plus Cow's Milk ELISA was developed for the detection of cow's milk, however it has also high cross-reactivity with buffalo's milk. There is no cross-reactivity with goat's, sheep's, horse's, donkey's and camel's milk.
The epitope recognised by the antibody used in the Plus Cow's Milk ELISA is heat stable, therefore the test is applicable for the detection of both raw and heat-treated cow's milk (pasteurised, UHT, milk powders).

The LOD, CCβ and cut-off were established with 3 batches of the Plus Cow's Milk ELISA kit under optimal conditions.

Application	Limit of detection (LOD) (% of cow's milk)	Cut-off value (% of cow's milk)	Detection capability (CCβ) (% of cow's milk)
Detection of cow's liquid milk in goat's/sheep's liquid milk (v/v)	0.2	0.3	0.5
Detection of cow's milk powder in goat's/sheep's milk powder (w/w)	0.2	0.3	0.5
Detection of cow's colostrum powder in goat's whey powder (w/w)	0.2	0.4	0.5

If the sample is found to be non-compliant, the results shall be verified by reanalysis 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.

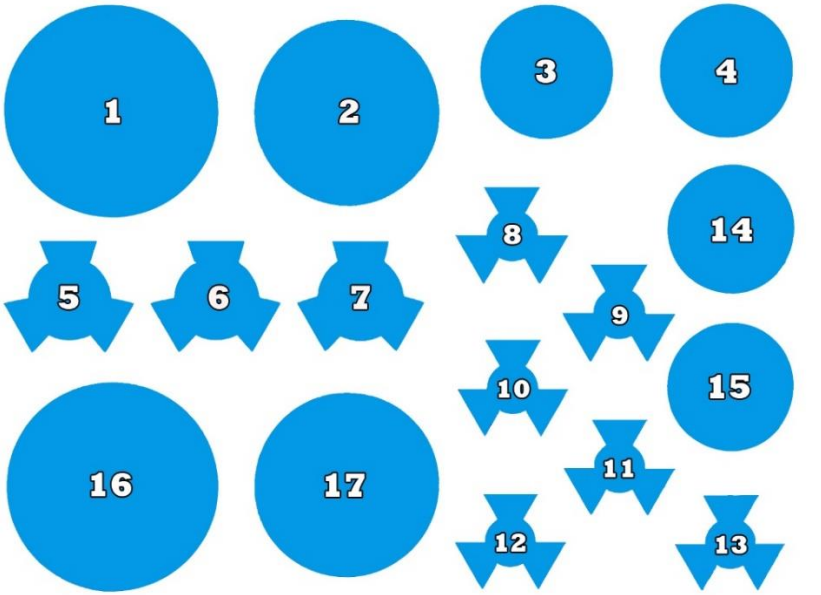
Degeneration of the reagents may have occurred when the following phenomena are observed:

- 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 (96-wells) microtiter plate (12 strips, 8 wells each), coated with κ-casein. Ready-to-use.

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



1. **Dilution buffer** (50 ml, ready-to-use)
2. **Rinsing buffer** (30 ml, 20× concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (12 ml, ready-to-use)
5. **Standard** (lyophilized)
6. **Standard** (lyophilized)
7. **Standard** (lyophilized)
8. **Mab-HRP Conjugate** (100 µl, 100× concentrated)
9. Not in 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 **Dilution buffer** (50 ml, ready-to-use)
17. Not in use
18 Not in use
19 Not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Vortex
- Rotary mixer
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Deionised istilled water (bidest)

7. PRECAUTIONS

- The stop reagent contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin.
- 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.
- TMB is toxic by inhalation, in contact with skin and if swallowed; take care when handling the substrate.
- 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, which crystallises 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

8.1. Detection of cow's liquid milk in goat's/sheep's liquid milk

- Dilute the milk 100 times in the dilution buffer in a following way:
    - pipette 50 µl of the milk sample and add 450 µl of the dilution buffer (1:10 diluted sample)
    - mix by vortex for 5 s
    - pipette 50 µl of the 1:10 diluted milk sample and add 450 µl of the dilution buffer (1:100 diluted sample)
    - vortex for 5 s
- Use 50 µl of the 100 diluted milk sample per well in the ELISA test

8.2. Detection of cow's milk powder in goat's/sheep's milk powder

- Reconstitute the milk by adding 1.3 g of milk powder to 9 ml of deionised water
- Vortex for 30 s and mix on a rotary mixer for at least 15 min
- Dilute the milk 100 times in the dilution buffer in a following way:
    - pipette 50 µl of the milk sample and add 450 µl of the dilution buffer (1:10 diluted sample)
    - mix by vortex for 5 s
    - pipette 50 µl of the 1:10 diluted milk sample and add 450 µl of the dilution buffer (1:100 diluted sample)
    - vortex for 5 s
- Use 50 µl of the 100 diluted milk sample per well in the ELISA test

8.3. Detection of cow's colostrum powder in goat's whey powder

- Weigh 1 g of goat's whey powder and add 9 ml of deionised water
- Vortex for 30 s and mix on a rotary mixer for at least 15 min
- Dilute the reconstituted sample 100 times in the dilution buffer in a following way:
    - pipette 50 µl of the sample and add 450 µl of the dilution buffer (1:10 diluted sample)
    - mix by vortex for 5 s
    - pipette 50 µl of the 1:10 diluted sample and add 450 µl of the dilution buffer (1:100 diluted sample)
    - vortex for 5 s
- Use 50 µl of the 100 diluted sample per well in the ELISA test

9. PREPARATION OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at +2ºC to +8ºC. Prepare reagents fresh before use.

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

Dilution buffers
This ELISA kit contains two bottles of dilution buffer which is ready to use (chapter 5, no .1 and 16. The dilution buffer is used for the dilution of the conjugate, standards and samples.

Standards
Prepare a dilution range of the standards. Add 1 ml of the dilution buffer to the lyophilised standard and mix. This solution contains κ-casein at the concentration corresponding to 4% of cow's milk (v/v or w/w) in the extracted samples when the procedures from chapter 8 are followed. Pipette 0.25 ml of this solution into a clean tube and add 0.25 ml of the dilution buffer. Continue to make a dilution range of 4, 2, 1, 0.5, 0.25 and 0.125% of cow's milk (v/v or w/w).

Conjugate solution
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min, 1000 x g). Add 10 µl of the concentrated conjugate solution to 990 µl of dilution buffer. Per 2 x 8 wells 800 µl of diluted conjugate is required. Store unused concentrated conjugate at +2ºC to +8ºC.

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

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISAs, between each immunological incubation step, unbound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
Basically, 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. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, 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 zero standard in duplicate (wells G1, G2, blank).
   Pipette 50 µl of the zero standard 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. 0.125, 0.25, 0.5, 1, 2 and 4%).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).

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

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

6. Incubate for 30 minutes in the dark at room temperature (20ºC to 25ºC)

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 for 15 minutes at room temperature (20ºC to 25ºC).
10. Add 100 µl of stop solution to each well.
11. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells G1 and G2 (blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard (wells A1 and A2) and multiplied by 100. The zero standard (Bmax) is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

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

Calibration curve:
The values (percentage maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the percentage of cow's milk on logarithmic X-axis.
The percentage of cow's milk (v/v for liquid milk, w/w for milk powders) in the sample corresponding to the percentage maximal absorbance of each extract can be read directly from the calibration curve.

Data interpretation:

Detection of cow's milk (liquid/powder) in goat's/sheep's milk (liquid/powder) (sample preparation 8.1 and 8.2)
For the detection capability of 0.5% the cut-off was found to be 0.3% meaning that any sample giving a result of ≥0.3% in the Plus Cow's Milk ELISA test should be considered as positive as it can contain 0.5% or more of cow's milk (v/v for liquid milk and w/w for milk powder).

Example data interpretation for screening for the presence of cow's milk in goat's/sheep's milk at the level of 0.5% and higher:

Result by Plus Cow's Milk ELISA	Positive/Negative?
0.1%	Negative
0.2%	Negative
0.3%	Positive
0.5%	Positive
1.2%	Positive

Detection of cow's colostrum powder in goat's whey powder (sample preparation 8.3)
For the detection capability of 0.5% the cut-off was found to be 0.4% meaning that any sample giving a result of ≥0.4% in the Plus Cow's Milk ELISA test should be considered as positive as it can contain 0.5% (w/w) or more of cow's colostrum powder.

12. LITERATURE

Haasnoot W, Smits NG, Kemmers-Voncken AE, Bremer MG. (2004) Fast biosensor immunoassays for the detection of cow's milk in the milk of ewes and goats. Journal of Dairy Research 71(3):322-9.
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. Advances in Dairy Research, 2:118.

13. ORDERING INFORMATION

For ordering the Plus Cow's Milk ELISA kit, please use cat. Code 5171MILK.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.

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