Plus Cow’s Whey ELISA

5171WHEY[1]04.21

A competitive enzyme immunoassay for the detection of cow’s rennet whey in whey from other species

EuroProxima Plus Cow's Whey ELISA

A competitive enzyme immunoassay for the detection of cow's rennet whey in whey from other species

TABLE OF CONTENTS

The quality management system of R-Biopharm Nederland B.V. is ISO 9001:2015 certified

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 Whey ELISA kit, please use cat. Code 5171WHEY.

14. REVISION HISTORY

Not applicable

R-Biopharm Nederland B.V.
Beijerinckweg 18
NL 6827 BN Arnhem
The Netherlands

Tel: + 31 26 3630364

E-mail: info@r-biopharm.nl
Web-site: http://www.europroxima.com

BRIEF INFORMATION

The Plus Cow's Whey ELISA is a competitive enzyme immunoassay for the detection of cow's rennet whey in whey 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 Whey ELISA is a fast screening tool for the detection of cow's rennet whey residues in whey 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 rennet whey in goat/sheep's whey 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 rennet whey powder in goat/sheep's whey powder as a result of cross-contamination between production batches.

2. PRINCIPLE OF THE ELISA

The Plus Cow's Whey ELISA uses a specific mouse monoclonal antibody (mAb) raised against the glycomacropeptide (GMP) part of bovine milk protein κ-casein. The antibody 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 any bovine GMP present in the sample. Bovine GMP is released from cow's κ-casein present in milk during cheese production and it is present in cow's rennet whey fraction. 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 rennet whey concentration of 8% (v/v or w/w).

There is a correlation between the amount of detected GMP and the percentage of cow's rennet whey present in the sample. When a calibration series is prepared from the provided standard a semi-quantitation can be achieved in the range from 0.25 to 8% of cow's rennet whey (v/v or w/w). Please note that the calibration is based on cow's rennet whey containing lowest level of protein/solid in order to assure that a sample containing 0.5% of cow's rennet whey of any type can give a result above the cut-off value. If a sample is contaminated with cow's rennet whey containing higher levels of proteins/solids (concentrated forms) it can give an overestimated result when a standard sample preparation method described in this manual is followed. If the concentration of protein/solid in cow's rennet whey contamination is known, please contact us to receive an Application Note with further instruction on adjusting the sample preparation method to obtain more accurate quantification.

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 whey rennet in the sample.

3. SPECIFICITY AND SENSITIVITY

Plus Cow's Whey ELISA was developed for the detection of cow's rennet whey, however it has also high cross-reactivity with buffalo's rennet whey. There is no cross-reactivity with goat's, sheep's, horse's, donkey's and camel's whey.

The LOD, CCβ and cut-off were established under optimal conditions.

Application	Limit of detection (LOD) (% of cow's rennet whey v/v or w/w)	Cut-off value (% of cow's rennet whey v/v or w/w)	Detection capability (CCβ) (% of cow's rennet whey v/v or w/w)
Detection of cow's rennet whey containing low or unknown amount of solid in liquid goat's/sheep's whey	0.1	0.5	0.5
Detection of cow's rennet whey containing low or unknown amount of protein in goat's/sheep's powder whey	0.3	0.5	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.

- The measured concentration is above the cut-off (0.5%) so it means that the sample can contain >0.5% of cow's rennet whey powder with low protein content.
- However, if the sample is actually contaminated with cow's rennet whey containing medium or high protein content (concentrated forms) then the result of 0.6% can be related to a low-level contamination. It means that this sample can also be contaminated with cow's rennet whey with medium/high protein content at a very low level (below 0.5%).
- There is a probability of getting a positive result for samples containing low level (<0.5%) of high or medium protein cow's rennet whey powder but there is a very low probability of not detecting a positive sample (>0.5% cow's rennet whey of any type).

Example 2:

- An end-user wants to analyse a goat's whey powder sample.
- The protein content in the cow's rennet whey powder that might contaminate their samples is known. The sample comes from a factory that also produces concentrated cow's whey powder (containing >70% of protein). The cross-contamination can occur as the same production line is used to produce goat's and cow's rennet whey concentrates.
- The end-user should follow the sample preparation method for samples suspected of being contaminated with cow's rennet whey containing high protein content (please refer to the Application Note describing the sample preparation method with an additional dilution step).
- The result by ELISA is 0.1% of cow's rennet whey.
- It means that the samples should not contain >0.5% of cow's rennet whey powder with high protein content.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (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.

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

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

Data interpretation:

The Plus Cow's Whey ELISA is a semi-quantitative test. The sample preparation methods presented in this manual can be used to screen for the presence of any type of cow's rennet whey (low, medium or high protein/solid content). When these standard sample preparation methods are followed, samples containing concentrated (e.g. medium or high protein/solid content) cow's rennet whey can give a result above the cut-off value even if the actual concentration is lower than 0.5%. On the other hand the probability of getting a false negative results (result below the cut-off even though the samples in fact contains >0.5% of cow's rennet whey of any type) is very low when the standard sample preparation methods from the manual are followed.
In case the % of protein/solid in cow's rennet whey which can contaminate the goat's/sheep's sample is known, please contact R-Biopharm Nederland to receive an additional Application Note. This Application Note describes how to adjust the sample preparation method for samples possibly contaminated with cow's rennet whey with known protein/solid content. This approach allows for more accurate quantification and reduces the risk of obtaining a positive result for samples containing <0.5% of cow's rennet whey with medium and high protein/solid content.

Example 1:
- An end-user wants to analyse a goat's whey powder sample.
- The protein content in the cow's rennet whey powder that can contaminate his samples is unknown.
- The standard method (8.2) should be followed using dilution factor of 10 after reconstitution of the whey powder.
- The result by ELISA is 0.6% of cow's rennet whey.

4. HANDLING AND STORAGE

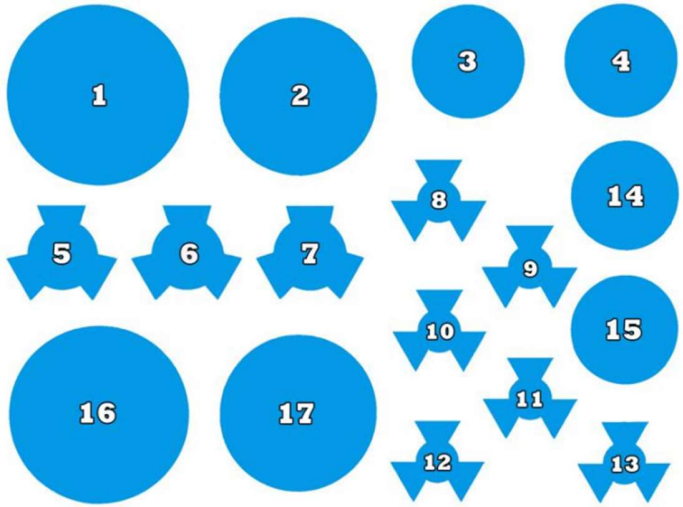
- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate 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

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.

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 dilution buffer in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the dilution buffer in duplicate (wells A1, A2, maximal signal).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.25, 0.5, 1, 2, 4 and 8%).

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 blank H1 and H2.

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)

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
- Distilled water (bidest)

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

8.1. Detection of cow's rennet whey with low (5%) or unknown solid content in goat's/sheep's liquid whey*

- Dilute the sample 8 times in the dilution buffer in a following way:
  - pipette 125 µl of the whey sample and add 875 µl of the dilution buffer (1:8 diluted sample)
  - mix by vortexing for 5 s
- Use 50 µl of this solution in the ELISA test

8.2. Detection of cow's rennet whey with low (11-15%) or unknown protein content in goat's/sheep's whey powder*

- Reconstitute the sample by adding 1 g of whey powder to 9 ml of deionised water
- Vortex for 30 s and mix on a rotary mixer for at least 15 min
- Dilute the sample 10 times in the dilution buffer in a following way:
  - pipette 100 µl of the sample and add 900 µl of the dilution buffer (1:10 diluted sample)
  - mix by vortexing for 5 s
- Use 50 µl of this solution in the ELISA test

*These are the standard sample preparation methods for the detection of cow's rennet whey of any type (low, medium or high protein/solid content). If the % of protein/solid in cow's rennet whey which can contaminate a sample is known, please contact R-Biopharm Nederland to receive an Application Note. The Application Note describes how to adjust the sample preparation method for samples possibly contaminated with cow's rennet whey with known protein/solid content. This approach allows for more accurate quantification.

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. Also retain the strip holder.

Dilution buffers
This ELISA kit contains two bottles of dilution buffer which is ready to use. 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 8% of cow's rennet whey (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 8, 4, 2, 1, 0.5 and 0.25% of cow's rennet whey (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.