PLUS MILK FRAUD / BOVINE RENNET WHEY ELISA

5171BRW[7]08.23

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

EUROPROXIMA PLUS MILK FRAUD / BOVINE RENNET WHEY ELISA

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

TABLE OF CONTENTS

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

5171BRW[7]08.23

BRIEF INFORMATION

The Plus Milk Fraud/BRW ELISA is a competitive enzyme immunoassay for screening of the presence of added bovine rennet whey powder (BRWp) to bovine milk or milk of other species and sources. The ELISA kit contains pre-coated 96-well microtiter plate and all the reagents to perform the assay. Samples and standards are measured in duplicate which means that a total of 41 samples can be analyzed with one kit.

1. INTRODUCTION

Bovine rennet whey (BRW) is a low-price by-product of cheese production from bovine (cow and buffalo) milk. It is traded mostly as powder (BRWp). Due to its low price, fraudulent mixing with the higher priced bovine milk and especially with the milk of other species (e.g. goat, sheep, camel, donkey, horse, etc.) and sources (e.g. soy, almond, barley, oat, coconut, etc.) is economically attractive. However, this is illegal, dangerous for allergic consumers and can lead to quality problems in end products such as yoghurt and infant formulas. Addition of BRW to dairy and other products can be identified by detecting the presence of the bovine glycomacropeptide (GMP), also named casein glycomacropeptide (CMP), which is released from bovine κ-casein by enzymatic (rennet) hydrolysis during cheese making process. The BRW ELISA uses a specific monoclonal antibody which recognizes a 5 amino acids-containing epitope on the GMP part of bovine κ-casein (Haasnoot et al., 2014; Bremer et al., 2008; Haasnoot, et al., 2004).
For the detection of BRWp in milk of other than bovine species and sources, milk samples are analysed directly after dilution in buffer. The limits of detections (LOD) for BRWp are 0.03% and 0.3% for 1:100 and 1:1000 diluted samples, respectively.
For the detection of adulteration of bovine milk with BRWp, the bovine κ-casein needs to be removed by precipitation with trichloroacetic acid (TCA) before the analysis. The GMP-containing neutralized supernatant is analysed after dilution in buffer which results in an LOD of 0.2% BRWp.

2. PRINCIPLE OF THE PLUS MILK FRAUD/BOVINE RENNET WHEY ELISA

The principle of the test is based on the binding of a HRP labeled monoclonal antibody (mAb) directed against an epitope on the GMP part of 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 bound GMP on the plate and free GMP in the samples or standards.
The amount of mAb-HRP bound to the GMP 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 the addition of sulphuric acid. The colour intensity is measured photometrically at 450 nm and it is inversely proportional to the concentration of GMP from the BRWp in the sample.

3. SPECIFICITY AND SENSITIVITY

The mAb applied in the PLUS Milk Fraud/BRW ELISA recognises a small 5 amino acids-containing epitope on the GMP part of bovine k-casein which is similar in cow's and buffalo's milk but it is not present in the milk of other species and sources (Haasnoot *et al*., 2014). The GMP is released during cheese making due to the enzymatic hydrolysis of κ-casein by added rennet. The applied mAb can recognise bovine κ-casein as well as the GMP released from the bovine κ-casein.

Measurement range

For the detection of BRWp in milk of other species and sources the sensitivity depends on the applied dilution. The measurement range is from 0.03% to 0.75% BRWp for 1:100 diluted samples.

For the detection of BRWp in bovine milk, the measurement range is from 0.2% to 4.25% BRWp, which can be adjusted by applying higher dilutions.

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:

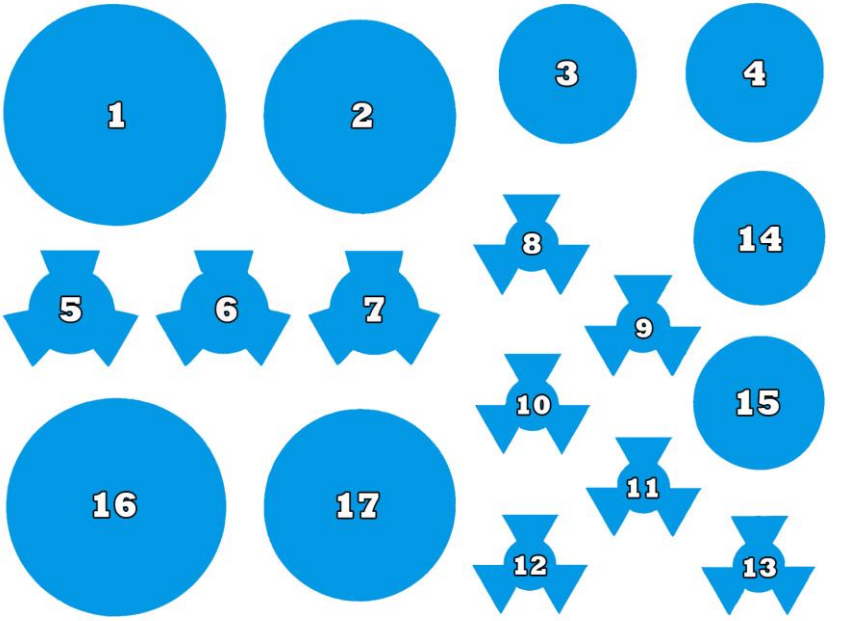
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

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

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



1. **Sample dilution buffer** (60 ml, ready-to-use)
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** (150 µl, 100 times concentrated)
9. Not in use
10. Not in use
11. Not in use
12. Not in use
13. Not in use
14. **Dilution buffer** (15 ml ready-to-use)
15. **Buffer A** (15 ml, 15x concentrated)
16. **SEB buffer** (ready-to-use)
17. Not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- 10 ml glass tubes
- Homogeniser
- Vortex mixer
- 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
- Syringe filters (PTFE-45/25; 0.45 µm)
- Syringes
- Aluminium foil or parafilm
- Distilled water
- Centrifuge
- TCA (50% solution in distilled water)

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 Bovine milk

Milk powder: *Prepare "milk"*
- *Add 1 g of milk powder to 9 ml distilled water*

- Homogenize the sample
- Pipette 2 ml of the homogenized milk into a tube (10 ml), add 3.8 ml of diluted buffer A and vortex for 30 seconds
- Add 1.2 ml of 50% TCA to the sample and vortex immediately for 30 seconds
- Centrifuge at 2000 x g for 10 minutes
- Filter through a syringe filter
- Dilute the filtrate 10x in SEB buffer (100 µl + 900 µl), vortex
- Dilute 1:1 with sample dilution buffer (200 µl + 200 µl), vortex
- Use 50 µl of this solution in the ELISA test.

8.2 Milk of other species (e.g. sheep or goat) and sources (soy):
- Homogenize the sample
- Pipette 10 µl of sample in 990 µl sample dilution buffer (1:100 dilution), vortex
- Use 50 µl of this solution in the ELISA test.

If the measured concentration of κ-casein exceeds 2.5 µg/ml a further dilution in sample dilution buffer is advised.
- Dilute once again 1:10 with sample dilution buffer (1:1000 dilution), vortex
- Use 50 µl of this solution 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.

Dilution buffer
For dilution of the concentrated conjugate.
Store at 4°C until the expiry date stated on the kit label.

Sample dilution buffer
The sample dilution buffer for dilution of the standard and samples.
The sample dilution buffer can be stored at 2ºC to 8ºC.

Standards
Prepare the standards freshly before use
Add 2 ml of the sample dilution buffer to a lyophilised standard vial and mix. This solution contains κ-casein at the concentration of 2,5 µg/ml. Prepare the dilution range in a following way:

1 µg/ml	200 µl of standard 2,5 µg/ml + 300 µl of sample dilution buffer
0,5 µg/ml	250 µl of standard 1 µg/ml + 250 µl of sample dilution buffer
0,25 µg/ml	250 µl of standard 0,5 µg/ml + 250 µl of sample dilution buffer
0.1 µg/ml	200 µl of standard 0,25 µg/ml + 300 µl of sample dilution buffer

SEB buffer
SEB buffer tends to precipitate at 4ºC. Make sure that this vial is at room temperature and mix the content well before use. If the crystals are visible, dissolve them by placing the buffer in a water bath at 37ºC.
Store unused buffer at room temperature.

Buffer A
Dilute buffer A 15x with distilled water (1 ml buffer + 14 ml distilled water).
Store unused diluted buffer at room temperature.

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 dilution buffer (chapter 5, no. 19). Per 2 x 8 wells 800 µl of diluted conjugate is required.
Store unused concentrated conjugate at 2°C to 8°C.

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).
Store at 4°C until the expiry date stated on the kit label.

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.

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 zero standard in duplicate (wells G1, G2, blanks).
   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. 0.10, 0.25, 0.50, 1.00 and 2.50 µg/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (41 samples; 82 wells).

4. Pipette 50 µl of conjugate (dilution chapter 9) 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 THE 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 to obtain the corrected absorbance values.

Qualitative interpretation

8.1 Bovine milk.
Milk samples giving average corrected absorbance values **higher** than the average corrected absorbance value obtained for the lowest standard (0.1 µg/ml) are considered to be **negative**, i.e. they contain <0.2% of cow's and/or buffalo's BRWp.
Milk samples giving average corrected absorbance values **lower** than the corrected absorbance value obtained for the lowest standard (0.1 µg/ml) are considered to be **positive**, i.e. they contain >0.2% of cow's and/or buffalo's BRWp.

The lower the corrected absorbance value of the sample, the higher the concentration of BRWp in the sample.

8.2 Milk of other species e.g. sheep or goat and sources (soy)
Milk samples (1:100 diluted):
- resulting in average corrected absorbance values **higher** than the average corrected absorbance value obtained with the lowest standard (0.1 µg/ml) are considered to be **negative**, i.e. they contain <0.03% of cow's and/or buffalo's BRWp.
- resulting in average corrected absorbance values **lower** than the average corrected absorbance value obtained with the lowest standard (0.1 µg/ml) are considered to be **positive**, i.e. they contain >0.03% of cow's and/or buffalo's BRWp.

Milk samples (1:1000 diluted):
- resulting in average corrected absorbance values **higher** than the average corrected absorbance value obtained with the lowest standard (0.1 µg/ml) are considered to be **negative**, i.e. they contain <0.3% of cow's and/or buffalo's BRWp.

- resulting in average corrected absorbance values **lower** than the average corrected absorbance value obtained with the lowest standard (0.1 µg/ml) are considered to be **positive**, i.e. they contain >0.3% of cow's and/or buffalo's BRWp.

Note I: The ELISA detects a small epitope on the GMP part of bovine κ-casein and, as such, detects both, GMP (present in BRWp) and whole κ-casein (present in bovine milk). Thus, a sample prepared according to chapter 8.2 and found positive by BRWp ELISA can contain bovine milk instead of BRWp. The sample preparation for bovine milk (chapter 8.1) can differentiate between added BRWp and bovine milk. If the same sample is negative after this preparation, it contains bovine milk. If the sample is positive after this preparation, it contains BRWp.

Semi-quantitative interpretation

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.

O.D. standard (or sample)
---------- x 100 = percentage of the maximal absorbance
O.D. zero standard (Bmax)

The values (percentage 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 (Figure 2).
In case of concentrations >2.5 µg/ml, samples need to be re-analysed after an additional dilution for semi-quantitative interpretations. The linear correlation between the amount of detected κ-casein and percentage of added BRWp depends on the sample preparation and dilution (see chapters 8.1 and 8.2).

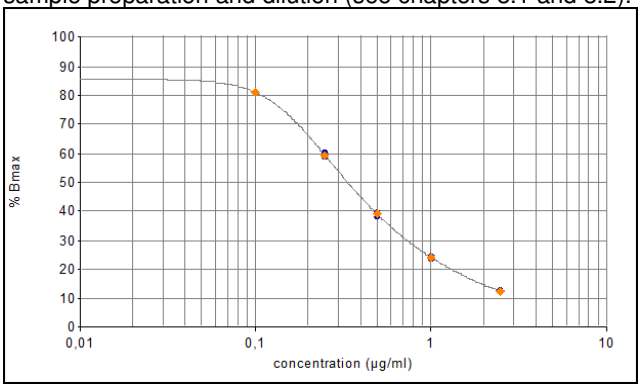


Figure 1 : Example of a calibration curve obtained with the ready-to-use standard solutions of bovine κ-casein supplied in the BRW ELISA kit.

8.1 Bovine milk

The percentages of BRWp in the bovine milk samples can be calculated as follows:

% BRWp = Concentration of κ-casein (µg/ml) x 1.7*

Measurement range from 0.2 to 4.25% BRWp.

Note II: Due to differences in the concentrations of κ-casein in different BRW powders, the calculated percentages of BRWp are indicative only.

8.2 Milk of other species (e.g. sheep or goat) and sources (e.g. soy)

The percentages of BRWp in the milk samples can be calculated as follows:

(1:100) diluted samples: % BRWp = Concentration of κ-casein (µg/ml) x 0.3*

Measurement range from 0.03 to 0.75% BRWp.

(1:1000) diluted samples: % BRWp = Concentration of κ-casein (µg/ml) x 3*

Measurement range from 0.3 to 7.5% BRWp

* Explanation of the multiplication factors:

8.1 The multiplication factor was calculated to be 1.7 for bovine milk samples.

8.2 The multiplication factors were calculated to be 0.3 (1:100 diluted samples) and 3 (1:1000 diluted samples) for milk from other species and sources.

The multiplication factors can easily be established after analyzing spiked milk samples

For 1:100 diluted milk samples of other species and sources, it is recommended to spike with 0.2% BRWp and for 1:1000 diluted milk samples it is recommended to spike with 2% BRWp.

For bovine milk samples it is recommended to spike with 1% BRWp.

The multiplication factors can be calculated as follows:

$$\frac{\text{percentage BRWp spiked (0.2 or 2 or 1)}}{\text{measured κ-casein concentration (µg/ml)}} = \text{multiplication factor (mf)}$$

See note I

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. Journal of Dairy Research 71(3), 322-329.

13. ORDERING INFORMATION

For ordering the Plus Milk Fraud/BRW ELISA kit, please use cat. Code 5171BRW.

14. REVISION HISTORY

The standard in this kit is changed from ready to use to a lyophillised form, because of stability reasons, see chapter 5 and 9.

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

Tel: + 31 26 3630364
www.europroxima.com
info@r-biopharm.nl