biosensis® Bovine A2 β-Casein ELISA Kit

Catalogue Number: BEK-2244-1P/2P

For the quantitative determination of bovine A2 β-casein level in bovine milk samples only when used as directed.

Please refer to the Sample Preparation Section for specific use instructions in bovine milk.

For research use only, not for use in clinical and diagnostic procedures.

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1. Intended Use

The purpose of this kit is the quantitative determination of Bovine A2 β-casein in bovine milk only if used as directed. The following milk samples have been tested successfully:

- A2 full cream milk
- A2 skim milk
- A2 powdered full cream milk (reconstituted in water)

This kit has not been tested for other sample applications. Non-validated samples may contain immunoglobulins and other components that potentially interfere with this ELISA assay. Biosensis does not assume responsibility if this kit is used for unintended purposes.

For research use only. Not for diagnostic and clinical purposes.

2. Introduction

Casein is encoded by the *CSN2* gene and consists of a 209-amino-acid single polypeptide chain, and molecular mass of about 24 kD. In cow's milk, caseins represent four gene products named αS1-, αS2-, β- and κ- casein, respectively. The relative concentration of these four caseins in milk can vary with different factors such as the breed, the lactation stage of the cow and the feeding.
In bovine species, total β-casein accounts for 9–11 g/L of skim milk and is characterized by genetic polymorphism (Farrel *et al*., 2004). β-casein is expressed as 13 genetic variants resulting from single nucleotide polymorphisms in the *CSN2* gene. The most frequent genetic variants in western dairy breeds are β-casein A1 and β-casein A2. The two types of beta-casein protein, A1 and A2, differ by a single-point mutation at amino acid position 82 (P82/H82).

The Biosensis Bovine A2 β-casein enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the quantification of Bovine A2 β-casein in 5 hours. This kit consists of a pre-coated rabbit anti-bovine β-casein polyclonal capture antibody, a chicken anti-bovine A2 β-casein detection antibody and a horseradish peroxidase (HRP)-conjugated donkey anti-chicken IgY antibody. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a colored reaction product which is directly proportional to the concentration of Bovine A2 β-casein present in samples and protein standards.

This Bovine A2 β-casein ELISA kit employs a purified, native A2 β-casein protein as a standard. This ELISA assay does not cross-react with Bovine A2 β-casein proteins.

3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
Bovine β-casein antibody coated 96-well microplate	12 x 8 wells	24 x 8 wells
Assay diluent B (1x)	2 x 25 mL	4 x 25 mL
Bovine A2 β-casein standard	2 x 200 ng	4 x 200 ng
Bovine A2 β-casein specific detection antibody	1 vial	2 vials
Anti-Chicken IgY-HRP conjugate (100x)	1 x 110 µL	2 x 110 µL
Wash buffer (10x)	1 x 33 mL	2 x 33 mL
TMB substrate (1x)	1 x 11 mL	2 x 11 mL
TMB stop solution (1x)	1 x 11 mL	2 x 11 mL
Plate sealer	Supplied	

Reagent	Storage and Stability
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard	Use on same day; aliquot unused protein to prevent multiple freeze-thaw cycles and store at -20°C for 2 weeks
Reconstituted detection antibody (100x) and diluted detection antibody (1x)	1 week at 2-8°C
Diluted HRP conjugate (1x)	2 weeks at 2-8°C
Diluted wash buffer (1x)	2 weeks at 2-8°C

Note:

- Do not freeze the HRP conjugate
- Do not use kit components from other ELISA kits

4. Equipment and Reagents Required but Not Supplied

- Single- and multi-channel pipettes capable of delivering 10-1000 µL volumes
- Plastic and glass ware for sample collection, sample preparation and buffer preparation
- Plate shaker
- Microplate reader capable of reading absorbance at 450 nm
- NaOH, 0.5 M (for dilution of milk samples)

5. Before You Start….

- Read the entire protocol to familiarize yourself with the assay procedure; a tutorial about the use of *Rapid*™ ELISA kits is available online at: https://www.youtube.com/watch?v=7EOuc9qYL0E
- Bring the microplate and all reagents and solutions to room temperature before starting the assay
- Remove only the required amount of TMB substrate from the fridge to prevent multiple warm-ups and cool downs; keep the TMB substrate in the dark
- Remove the number of strips required and return unused strips to the pack and reseal
- Centrifuge all kit components to collect reagents to the bottom of tubes before use
- The stop solution provided with this kit is an acid; wear protective equipment when handling
- Please visit our Technical Notes section at www.biosensis.com for further information and helpful hints on ELISA-related topics

6. Sample Preparation

In general, caseins are not soluble at neutral pH. A 1:100 dilution in 0.5 M NaOH (10 µL of milk sample with 990 µL 0.5 M NaOH) needs to be performed before further dilutions are carried out in Assay Diluent B. It is absolutely essential that the samples are vortexed vigorously on each dilution.

The A2 milk samples that were tested in our laboratories show parallelism and thus accurate quantification in the 1:40,000 to 1:640,000 dilution range (total dilution taking into account the primary 1:100 dilution step with 0.5 M NaOH). Note that the concentration of Bovine A2 β-casein varies between the processes to which the milk has been subjected (for example, homogenization, spray drying etc.). Therefore, we recommend that at least 3-4 dilutions per milk sample type are performed to determine the range in which the samples need to be diluted to ensure that their OD values are within the range of the standard curve.

Stability of Milk Samples

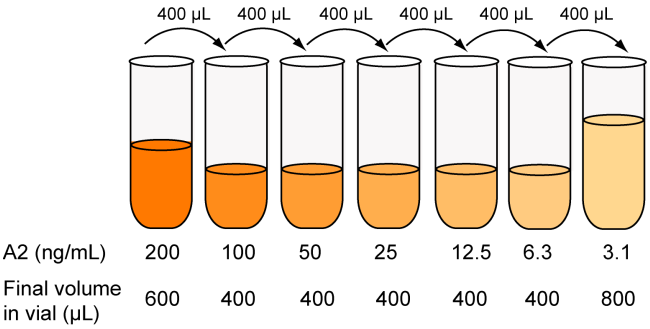
Only milk samples that have been stored at 2-8°C and powdered milk samples that have been reconstituted in water have been tested in this kit. The stability of milk samples stored at -20°C and -80°C has not been tested. Diluted milk samples for assaying should be prepared fresh and used within 2 hours of preparation.

7. Preparation of A2 β-Casein Standard

- Reconstitute the lyophilized antigen standard with 1 mL of Assay diluent B
- Label the vial with the reconstituted A2 standard as “200 ng/mL”; vortex and let stand for 15 minutes
- Note: 200 ng/mL is the highest concentration of the A2 standard curve. In order to generate the rest of the A2 standard curve, perform a 1:2 serial dilution down to 3.1 ng/mL.

The volumes used for the dilution series depends on the number of repeats per A2 β-casein concentration. For triplicate measurement (100 µL per well) of each A2 β-casein standard concentration, we recommend this procedure:

1. Label 6 tubes with “100 ng/mL” “50 ng/mL”, “25 ng/mL”, “12.5 ng/mL”, “6.3 ng/mL” and “3.1 ng/mL”, respectively
2. Aliquot 400 µL of the assay diluent into each tube
3. Take 400 µL from the “200 ng/mL” tube and transfer to the “100 ng/mL” tube
4. Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex
5. Repeat steps 3 and 4 for each consecutive concentration until the last tube “3.1 ng/mL” is prepared and mixed well



8. Other Reagents and Buffer Preparation

- Detection antibody (100x): reconstitute detection antibody vial with 110 µL Assay Diluent B to give a 100x concentrated stock solution. Then dilute one hundred fold with Assay diluent B to get a 1x working solution. Prepare enough volume to add 100 µL per well.
- Donkey anti-chicken IgY-HRP conjugate (100x): dilute one hundred-fold with Assay Diluent B and prepare enough volume to add 100 µL per well.
- Wash buffer (10x): crystals may form at the bottom of the bottle; equilibrate to room temperature, mix thoroughly and dilute ten-fold with ultrapure water; each 8-well strip requires about 25 mL of 1x wash buffer per assay

9. Assay Procedure

All steps are performed at room temperature (20-25°C, 70-75°F).

1. Allow all buffers and the ELISA plate to equilibrate to room temperature before beginning the assay.
2. Add 100 µL of diluted A2 β-casein standards, samples and blank (sample diluent only) to the pre-coated microplate wells
3. If available, include sample-specific negative and positive control samples in the assay procedure
4. Seal the plate (e.g., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 120 minutes
5. Discard the solution inside the wells and perform 5 washes with 1x wash buffer (200 µL per well). See the technical hints section for a detailed description of the washing procedure
6. Add 100 µL of the detection antibody (1x) into each well
7. Seal the plate (e.g., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 120 minutes
8. Discard the solution inside the wells and wash as described in step 4
9. Add 100 µL of the donkey anti-chicken IgY-HRP conjugate (1x) conjugate into each well
10. Seal the plate (e.g., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 60 minutes
11. Discard the solution inside the wells and wash as described in step 5
12. Add 100 µL of TMB into each well and incubate plate at room temperature for 10-20 minutes without shaking in the dark
13. Stop the reaction by adding 100 µL of the stop solution into each well. Visible blue color will change to yellow. Read the absorbance at 450 nm on a plate reader. **Note:** Color will fade over time; therefore, we recommend that the plate be read within 5 minutes of adding the stop solution or no longer than 30 minutes after addition.
14. See Appendix A for a troubleshooting guide when unexpected difficulties are encountered

* RCF= 1.12 x Orbit Radius x $(rpm/1000)^2$

10. Technical Hints

1. Do not perform dilutions within the well
2. At least duplicate measurements for each standard and sample dilution is recommended
3. Dilute samples to an A2 β-casein concentration that falls within the range of the standard curve. Do not extrapolate absorbance readings
4. Avoid touching the inside surface of the wells with the pipette tip
5. Proper emptying and washing of the plate is crucial for low background and removal of non-specific binding. For manual plate washing, we recommend the following procedure:
    a. To remove liquid from the wells, place the plate on the palm of the hand and quickly invert the plate over the sink. Forcefully move the arm downwards and stop abruptly to force the liquid out of the wells. When done correctly, this technique should prevent liquid from getting

onto the fingers or onto the outside of the microplate wells

b. Blot and forcefully tap the microplate against clean paper towels 3-5 times
c. Wash the wells by pipetting 200 µL of wash buffer into each well and empty the wells as described in step a-b
d. Repeat this procedure for a total of 5 times

6. Complete removal of liquid from the wells at each step is essential for reliable results. However, avoid letting the wells dry out at any time
7. Add TMB and the stop solution to the wells in the same order
8. Ensure that all bubbles are removed and that the bottom of the plate is optically clean prior to taking the absorbance reading
9. Stopping the TMB reaction after about 10 minutes is usually sufficient to obtain a standard curve. However, TMB incubation times may vary based on the differences in laboratory temperature, temperature of the diluents and incubation times. We use a plate shaker set to 140 rpm (0.351 G) and perform all incubations at room temperature (20-25˚C, 70-75˚F) in our laboratories. Importantly, we read our plates on a microplate reader capable of measuring up to an optical density (OD) of 4. You may need to adjust the TMB incubation time if your microplate reader has a more limited range.
10. TMB incubation times can vary between 10–20 minutes, and assay sensitivity can be affected if color development is stopped too early. In contrast, if the assay is allowed to overdevelop, it could lead to high blanks or colour intensity that exceeds the range of the plate reader. In order to determine the optimal time to stop the TMB incubation, blue color development should be monitored at **650 nm**. Addition of Stop Solution will convert blue into yellow color, with an ~2.5-3X increase in OD at **450 nm**. Once the highest standard reaches an OD of ~0.8 – 1.0 at 650 nm, immediately stop the reaction by adding Stop Solution. Note that before taking a 650 nm measurement, the plate should be briefly and gently shaken by hand to homogenise the blue TMB reaction product within the wells for most accurate readings.

11. Calculation of Results

1. Average the readings for each A2 β-casein standard concentration, blank and sample
2. Plot a standard curve with the A2 β-casein standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the A2 β-casein standards are adjusted for background absorbance, then subtract the blank value from the OD_{450} of the samples as well
4. Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit; avoid using linear regression analysis
5. Perform a 4-PL regression analysis to calculate the concentration of A2 β-casein in the samples. Multiply the result by the total sample dilution factor taking into account the dilution with sodium hydroxide solution

Manual Plate Reading

The relative OD_{450} = (the OD_{450} of each well) – (the OD_{450} of Zero well and/or Blank well).

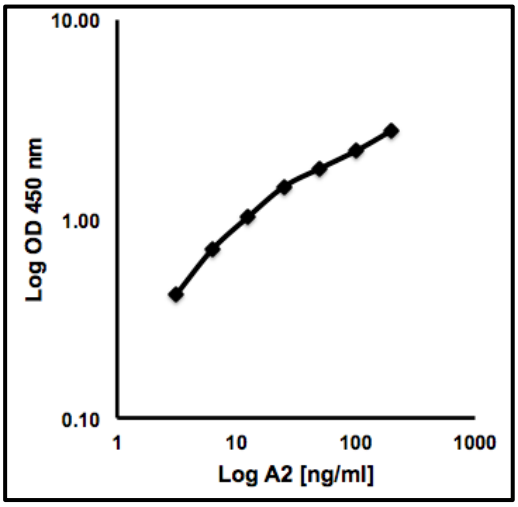
1. The **standard curve** can be plotted as the relative OD_{450} of each A2 β-casein standard solution (Y-axis) vs. the respective known concentration of the A2 β-casein standard solution (X-axis). The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding OD_{450}. The greater the concentration of target protein in the solution, the higher the OD_{450}.
2. **Determine concentration of target protein in unknown sample:** The A2 β-casein protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of A2 β-casein in the unknown sample. If the samples measured were diluted, multiply the concentrations from the interpolation with the dilution factor to obtain the actual A2 β-casein concentration in the sample.

12. Typical Data

Standard Curve

Standard curves are provided for demonstration only. A standard curve has to be generated for each A2 ELISA assay.

In addition, we recommend measuring common samples when performing multiple assays across several days in order to normalize the standard curve numbers between the various runs.



In the example graph above, A2 standards were run in triplicates using the assay diluent provided in this kit. The TMB reaction was stopped after 11 minutes.

Typical optical densities and coefficient of variations for diluted A2 standards are summarized in the following table:

A2 / [ng/mL]	OD 450nm	SEM	STDEV	%CV
200	2.772	0.025	0.043	1.5
100	2.192	0.045	0.078	3.6
50	1.790	0.014	0.025	1.4
25	1.450	0.034	0.059	4.1
12.5	1.022	0.010	0.018	1.8
6.3	0.70	0.022	0.038	4.2
3.1	0.416	0.036	0.063	15.1
Blank	0.199	0.011	0.020	10.0

SD: standard deviation; SEM: standard error of mean; CV: coefficient of variation

Limit of Detection

This Bovine A2 β-Casein ELISA kit detects < 3 ng/mL A2 β-casein in assay buffer (defined as A2 β-casein concentration at blank OD plus 3X standard deviations of the blank OD (n=10)).

Accuracy of A2 β-Casein : Quantification in Bovine Milk

Accuracy and possible matrix effects were assessed by performing parallelism experiments between the calibration standard curve (in Assay Diluent B) and serially diluted milk samples. The different kinds of milk samples used were:

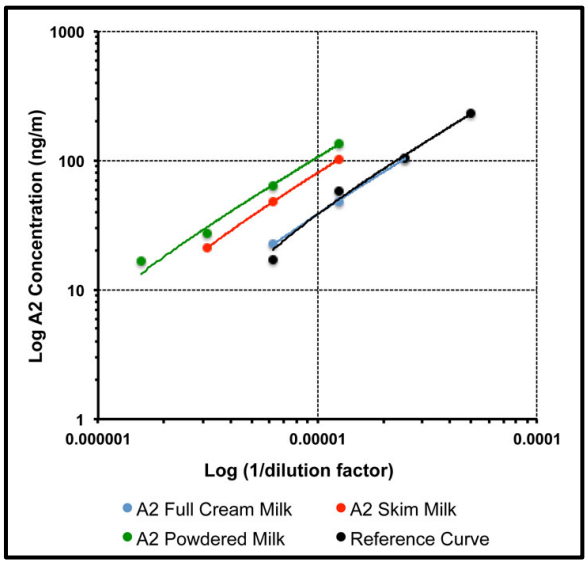
a) A2 full cream milk
b) A2 skim milk
c) A2 powdered milk (premium instant A2 full cream milk powder)

Each milk sample was diluted to three to four concentrations with Assay Diluent B after the initial dilution in 0.5 M NaOH. The precision between diluted samples in a dilution series ranged from 8.7 to 14 % which meets the European Medicines Agency (EMA) Bioanalytical Method Validation Guideline requirements for a CV of less than 20%.

Parallelism in Bovine Milk Samples			
Sample	**Dilution**	**A2 (ng/mL)**	**A2 (mg/mL) Dilution Corrected**
A	1/40,000	104	4.2
	1/80,000	49	3.9
	1/160,000	23	3.7
	Mean		**3.9**
	%CV		**6.4**
B	1/40,000	165	6.6
	1/80,000	102	8.2
	1/160,000	48	7.7
	1/320,000	21	6.7
	Mean		**7.3**
	%CV		**10.7**
C	1/80,000	135	10.8
	1/160,000	63	10.1
	1/320,000	28	8.8
	1/640,000	17	10.8
	Mean		**10.1**
	%CV		**9.3**

All samples showed detectable levels of A2 β-casein demonstrating that A2 β-casein levels can be detected in

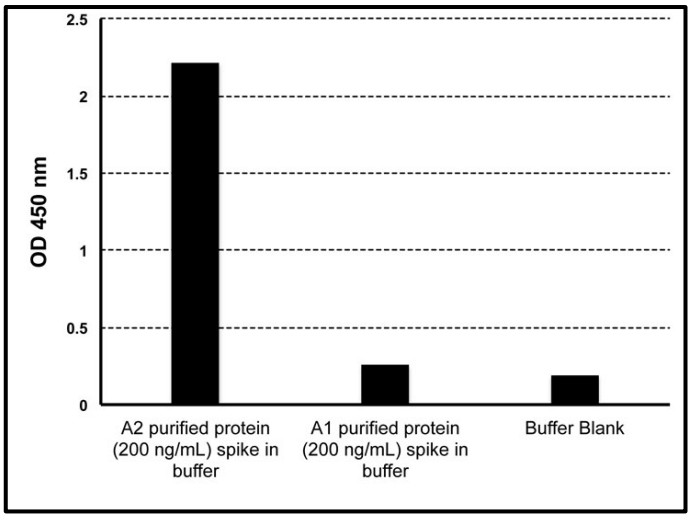
various forms of bovine milk and that the milk processing methods tested do not affect assay accuracy.



Note: the dilution range for the reference curve in the above graph has been adjusted to fall into the same range like for the milk samples for graphical purposes.

Specificity

Purified A2 and A1 β-casein proteins were spiked into 2 different aliquots of Assay Diluent B buffer at a concentration of 200 ng/mL. No cross-reactivity was observed with A1 β-casein protein.



13. Informational References

European Medicines Agency 2011, Guidelines on Bioanalytical Method Validation. 21 July 2011.

Farrell *et al.* 2004, *Nomencalture of the proteins of Cow's Milk- Sixth Revision.* Journal of Dairy Science June ;87(6):1641-1674.

Swaisgood H.E 1992, *Chemistry of the caseins* in Fox P.F, editor. Advanced Dairy Chemistry 1st ed. Volume 1. Elsevier Applied Science; London, U.K.: 63–110.

14. Other Information

Please visit our website (www.biosensis.com) for a full range of A2 β-casein and A2 β-casein related research reagents.

Appendix A: Troubleshooting Guide

This Bovine A2 β-casein ELISA kit has been developed to deliver reproducible results when following the provided assay protocol. Please refer to the troubleshooting guide below when unexpected difficulties are encountered. If you require further assistance, talk to a scientist at Biosensis (biospeak@biosensis.com).

Problem	Cause	Solution
High background (blank OD > 0.30)	Insufficient washing	Make sure to perform 5 washes after each incubation step with sample, detection antibody and streptavidin-HRP conjugate. Follow the recommended manual washing procedure for optimal washing performance
	Excessive concentration of detection antibody and/or HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume.
	Increased incubation time and temperature	Please follow incubation times as stated in the protocol and perform incubations at room temperature
	Contamination	Ensure that all plastic- and glassware used is clean. Do not use the same pipette tip to transfer samples and blank solution into the wells
Low absorbance readings	Concentration of A2 in the sample is lower than the detection limit of this assay	Confirm that the assay protocol was carried out correctly by spiking a known amount of A2 β-casein into your sample and/or check that the QC sample value falls within the expected A2 concentration range
	Insufficient antibody or insufficient HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume
	Reagents expired	Ensure correct storage of the kit and do not use the kit beyond its expiry date
Low absorbance readings	Decreased incubation times and temperature	Please follow incubation times as stated in the protocol and warm reagents to room temperature before use
	Microplate wells dried out	Do not leave microplate wells without solution for an extended time
	Wash buffer not diluted	Ensure that the wash buffer is diluted with ultrapure water to a 1x working solution
	Stop solution not added	Add the stop solution to obtain a yellow reaction product that can be measured at 450 nm
Standard OD values above plate reader limit	Excessive incubation with TMB substrate solution	Reduce incubation time by stopping the reaction at an earlier time-point
Sample OD values above standard curve range	A2 β-casein concentration in sample is too high	Perform a serial dilution of your sample to obtain a reading that falls within the standard curve
High coefficient of variations (CV)	TMB and stopping solution not added consistently	Add TMB and stopping solution to the wells in the same order and speed
	Inconsistent pipetting	Ensure pipettes are working correctly and are calibrated; review your pipetting technique; add standards and samples to the wells using a single-channel rather than a multi-channel pipette
	Insufficient mixing of reagents	Briefly vortex to mix solutions before pipetting into the wells
	Bottom of the plate is dirty	Clean the bottom of the plate before reading the plate