biosensis® Customer Built Bovine A2 Beta Casein ELISA Reagent Set Protocol (5 or 10 plates)

Catalog No: BEK-2365-SET5/SET10

For quantitative detection of bovine A2 beta casein level in bovine milk samples only when used as directed.

This is a reagent set for a customer-built ELISA assay against Bovine A2 Beta Casein making either 5 x / 10 x 96-well ELISA assays

IMPORTANT: Please store all four components at -20 °C. They will be expired in 12 months from date of receipt.

For research use only, not for diagnostic application.

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I. Experimental Overview

The Biosensis Bovine A2 Beta Casein ELISA assay has been designed for the quantitative detection of bovine A2 beta casein level in bovine milk samples only when used as directed. The principal of the ELISA is a sandwich assay. Affinity-purified rabbit anti- beta casein polyclonal antibodies (pAbs) are coated on ELISA plate. A2 beta casein standards (6.25 ng/mL- 400 ng/mL) and A2 beta casein protein in tested samples will be captured, and the A2 specific chicken pAbs will be used to detect the A2 beta casein. Rabbit anti-chicken IgY HRP conjugate will be used for signal amplification. After color development, OD450 will be measured and A2 standard curve will be established. Concentration of A2 beta casein in tested sample will be estimated based on the standard curve. In general, house milk samples need to be diluted to 1:10,000 to 1:50,000 since A2 level are high. For testing A2 contamination in A1 milk, sample can be diluted to 1:10-1:1,000.

For each plate, it is recommended that two columns will be used for standard curve establishment with the detecting range of 6.25 ng/mL to 400 ng/mL A2 and that samples be run in duplicate at least.

Assay Range: 6.25 ng/mL-400 ng/mL; Specificity: Bovine casein A2 protein, no cross reactivity detectable with A1 allele.

Note: Biosensis product BEK-2365-SET5/SET10 is designed as a set of antibodies & standards for a customer-built ELISA assay against Bovine A2 Beta Casein making either 5 x / 10 x 96-well ELISA assays. Customers will need to add additional components including plates, buffers, and detection substrates to enable a completely functional assay.

II. Materials Provided

Reagent Component	Unit Size/ Volume	Quantity (5 Plates)	Quantity (10 plates)
Rabbit anti-beta casein Ab for coating A2 plates	1 mg/mL	1 tube (500 ug)	1 tube (1000 ug)
A2 beta casein standard (40 µg/mL (40 ng/uL)	20 µL/tube (40 µg/mL)	5 tubes*	10 tubes*
Chicken anti-A2 beta casein specific Detection Antibody (300X)	40 µL/tube (0.3 mg/mL)	5 tubes*	10 tubes*
Rabbit Anti-Chicken IgY HRP conjugate (1000X)	20 µL/tube	5 tubes*	10 tubes*

*Each tube is enough for one ELISA plate

Please store all four components at -20 ^{o}C. They will be expired in 12 months upon receipt if unopened. **Important**: Before opening vials, spin vials briefly to collect antibody and protein at the bottom of the tube.

III. Equipment & Materials Required but Not Supplied

1. Automated plate washer is desirable
2. Multi-channel or repeating pipette
3. Clean tubes and Eppendorf tubes
4. Plate shaker (300-500 rpm)
5. Mechanical vortex
6. Microplate reader with 450 nm filter
7. Pipettors and tips
8. BSA (Fraction V, protease, IgG-free, low electrolyte recommended)
9. TBS and TBST (see following instruction)
10. ELISA coating buffer (see following instruction)
11. Tween-20 (Polyoxyethylene-Sorbitan Monolaurate)
12. Substrate TMBS (Recommended: Cat#: TMBUS-1000, Moss, Inc, www.mosssubstrates.com.
13. ELISA plates (Recommended: NUNC cat#468667)
14. HCl (Reagent grade), 1M for stopping reaction in final step
15. NaOH (ACS Grade), 0.5M for milk sample preparation

IV. Reagent & Plate Preparation Instructions

ELISA COATING BUFFER STOCK (1 L):

Na_2CO_3 (ACS Grade)	1.59 g
$NaHCO_3$ (ACS Grade)	2.93 g
D.I. Water (HQ)	1 L

Adjust pH to 9.5-9.7, filter via a 0.22 micron filter, store refrigerated for no more than a week in glass.

10X TBS BUFFER STOCK (1L):

NaCl (ACS grade)	24 g
KCl (ACS grade)	2 g
Trizma Base (ACS Grade)	12.1 g
D. I. Water to 1L	

1) Dissolve all dry reagents together in 800 mL of ddH2O
2) Adjust pH to 8.6 with 1M HCl
3) Add ddH2O to a final volume of 1 L, check pH
4) Filter through 0.22 micron filter or sterilize via autoclaving.

1X TBS BUFFER (1L): 10 mM Tris-HCl, ~40 mM NaCl, 27 mM KCl, pH 8.6

Add 100 mL 10X TBS into 900 mL D.I. water and mix well. Adjust pH to 8.6 with 1M HCl. TBS buffer (1X) may be filtered via a 0.22 micron filter if desired. Store refrigerated for no more than a week.

1X TBST BUFFER (1L): 10 mM Tris-HCl, ~40 mM NaCl, 27 mM KCl, pH 8.6, 0.05% Tween-20

Add 0.5 mL Tween-20 (Polyoxyethylene-Sorbitan Monolaurate) to 1 L TBS (1x) and mix well. TBST will be used for preparing the antibody dilution buffer and for plate washing. Prepare 1 L per plate.

BLOCKING BUFFER (20 ML PER PLATE): 5% BSA in TBS (1X)

Dissolve 1 g BSA in 20 mL TBS (1X) buffer.

BSA will dissolve slowly, thus extended stirring is required. Filtering through a 0.22 micron non-protein binding filter (eg., PES) is recommended. Store at 2-8^{o}C for no more than 3 days.

ANTIBODY AND SAMPLE DILUTION BUFFER (50 ML PER PLATE): 1% BSA in TBST

Dissolve 1 g BSA in 100 mL TBST (1X) buffer.

BSA will dissolve slowly, thus extended stirring is required. Filtering through a 0.22 micron non-protein binding filter (eg., PES) is recommended. Store at 2-8^{o}C for no more than 3 days.

PREPARATION OF COATED PLATES:

1. Prepare 10 mL of diluted antibody per plate. Dilute rabbit anti-beta casein pAbs to 10 ug/mL (1:100 dilution) in ELISA coating buffer and apply 100 uL/well. Store the plate at 2-8 ^{o}C overnight or for up to 3 days.

2. Empty coating solution from the wells and block with 200 uL/well Blocking buffer for at least one hour at room temperature. Refer to Technical Hints Section for instructions on emptying plates. Store at 2-8 ^{o}C for no more than 3 days if not using right away. Do not let the wells dry out. We have not yet tested this ELISA on dried and rehydrated plates, only freshly prepared wet plates as described. Use coated, blocked plates in step one of the Assay Procedure.

PREPARATION OF STANDARDS:

(a) 400 ng/mL A2 standard (tube #1): Add 10 uL of the 40 ug/mL (40 ng/uL) A2 stock protein standard into 990 uL Ab dilution buffer to make 400 ng/mL A2 standard. Mix gently. This is tube #1, the high standard.

(b) 200 ng/mL - 6.25 ng/mL standards: Label 6 additional Eppendorf tubes with (tube #2) 200 ng/mL, (tube #3) 100 ng/mL, (tube #4) 50 ng/mL, (tube #5) 25 ng/mL, (tube #6) 12 ng/mL, and (tube #7) 6.25 ng/mL, respectively. Aliquot 0.5 mL of antibody/sample buffer into tubes 2-7. Tube #1 already has the 400 ng/mL A2 standard in it from step (a). Add 0.5 mL from tube #1 tube into tube #2 and mix. Serially transfer 0.5 mL from 2nd tube into 3rd tube and mix. Continue performing serial dilutions through tube seven. In the end, tube#1 has 400 ng/mL, tube#2: 200 ng/mL, #3: 100 ng/mL; #4: 50 ng/mL; #5: 25 ng/mL; #6: 12.5 ng/mL; #7: 6.25 ng/mL.

Standard solutions should be prepared no more than 3 hours prior to the experiment. The working standard solution may be stored at 4°C for up to 3 hours. Do NOT FREEZE

PREPARATION OF DETECTION ANTIBODY:

Prepare 10 mL per plate. Dilute A2 specific detecting pAb (Chicken IgY) in Ab dilution buffer at ~ 1:300 dilution, ie, add 35 uL A2 Detecting Abs into 10 mL Ab dilution buffer, mix gently, use immediately. Prepare just before use. Do not store.

PREPARATION OF ANTI-CHICKEN HRP SECONDARY ANTIBODY:

Prepare 10 mL per plate. Dilute rabbit anti-chicken IgY HRP conjugate to ~ 1:1,000 in Ab dilution buffer (10 uL HRP conjugate into 10 mL Ab dilution buffer, mix well). **Prepare just before use. Do not store.**

V. Sample Preparation and Dilutions

In general, caseins are not soluble at neutral pH and thus the milk samples need to be diluted at 1:10-1:100 in 0.5M NaOH first (10 uL milk in 90 uL for 1:10, or 990 uL NaOH for 1:100), then, further diluted in antibody/sample dilution buffer for final sample use.

In general, for A2 detection in A2 milk (household organic milk), it can be additionally diluted to a total dilution of 1:10,000-1:50,000 for testing. For testing possible A2 contaminated A1 milk, you may dilute to a total dilution of 1:10-1:1,000 for testing. **Samples can be stored at 2-8°C for up to 3 hours; do not freeze.**

VI. Assay Procedure

We recommend that standard solution dilutions and each sample are plated in duplicate. Standard curves must be included on each plate, even if multiple plates are assayed in parallel. Read entire protocol before beginning. Prepare all reagents and test samples as instructed above.

1) Using the coated ELISA plate, prepared as directed in Section IV, empty the blocking solution from the wells and wash 3X with TBST, 1 minute each. Add 100 uL/well standards or prepared sample in duplicate and make sure to record each well location on a record sheet.

2) Mix the plate on an ELISA plate shaker at 400 rpm for 2 hour at RT; cover plate with parafilm or equivalent

3) Wash 3X with TBST, 2 minutes each.

4) While washing the plate, prepare (10 mL per plate) A2 specific detecting pAb (Chicken IgY) in Ab dilution buffer at ~ 1:300 dilution, (i.e., add 35 uL A2 Detecting Abs into 10 mL Ab dilution buffer)

5) Empty the last wash and add the prepared A2 detecting pAb at 100 uL/well to the plate and shake at 400 rpm for 2 hour at RT, covered.

6) Wash 3X with TBST, 3 minutes each.

7) While washing the plate, prepare Rabbit anti-Chicken IgY HRP conjugate (10 mL per plate) solution: dilute the HRP conjugate to ~ 1:1,000 in Ab dilution buffer (10 uL HRP conjugate into 10 mL Ab dilution buffer, mix well but gently, do not vortex).

8) Empty the last wash and add the prepared rabbit anti-chicken IgY-HRP conjugate 100 uL/well to the plate, cover.

9) Mix the plate on the shaker (400 rpm) for another 1 hour at RT

10) Wash 5X with TBST, 3-5 minutes each.

11) After the last wash, add 50 uL/well substrate (TMBS) and cover with light proof cover (foil recommended). Note the order and arrangement of the substrate addition when applying to the plate. One must add the stop solution in the same order for best results. In the dark, let the color to develop 4-10 minutes without shaking. **Color development can be quick**, check at 4, 6, 8 minutes; over development will just raise backgrounds.

12) Stop the reaction with the addition of 50 ul 1M HCl to each well, in the same pattern and order that was used to load the TMBS substrate. It is recommended to immediately read the plate at 450 nm with a microplate reader. Note: Color will fade over time; hence, we recommend plate to be read within 30 min after adding the stop solution. Immediate and 30 minute later readings may differ; this is normal for TMBS reactions. When doing multiple plates, try to read them all in a consistent manner.

CAUTION: Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.

13) Record the development time on the ELISA recording sheet along with OD450 readout. Make a standard curve using the average OD450 from the two reference standards for each point. Determine the A2 concentration for the tested samples based on their OD450 and standard curve. Make sure that the final concentration is obtained by multiplying with dilution factor.

VII. Technical Hints

1. Spin all liquid components to get liquid to the bottom of tubes before use.
2. Duplicate well assays are recommended for both standard and sample testing. All assay steps are performed at room temperature.
3. In order to avoid marginal effects of plate incubation due to temperature differences, it is suggested that the TMB solution be brought to room temperature.
4. Recommended method for manual plate washing.
    - **Emptying of wells on the plate**: Place the plate on the palm of the hand in a position that enables easy flicking movement using the wrist. Holding the plate over a sink, quickly invert the plate, whilst accelerating the arm downward toward the sink. Abruptly stop the downward acceleration to force the liquid from the wells into the sink. When done correctly the technique should prevent liquid from getting on to the fingers or on the outside of the strip wells or plate holder. *Note: Retain the upside down position of the plate to avoid any back flow into the wells. DO NOT LET THE PLATE DRY OUT.*
    - **Blotting the plate**: Immediately blot the inverted plate by lightly tapping the plate 3-4 times on blotting paper.
    - **Washing**: Forcefully pipette Wash Buffer into each well with a multi-channel pipette. Empty the wells of wash buffer using technique described above. Repeat washing and flicking procedures thrice. When washing the plate, each time let the washing buffer stay in the wells for 1-2 min before rinsing with more wash buffer as directed above. *Note: **Avoid** touching the inside surface of the wells with the pipette tips.*

Do not let the wells dry out at any time or assay performance will be poor

VIII. Reagent Storage

If the reagents will not be used with 1 week of receipt, then the -20°C components should be stored in -10°C to -20°C freezer. Expiration of the unopened set of reagents is 12 months if stored as directed below. Once opened, use all components within 1 week. Place components in appropriate temperature locations as directed below. For -20°C items multiple freeze-thaw cycles are NOT recommended. Divide into aliquots if not using all thawed material immediately.

Reagent Components	**Storage Temp**
Rabbit anti-beta casein Ab for coating (1 mg/mL)	-20°C, aliquot out into single use portions
A2 beta casein standard (40 µg/mL)	-20°C, single use tube; 1/plate; do not reuse
Chicken anti-A2 beta casein specific detection pAb antibody (0.3 mg/mL)	-20°C, single use tube; 1/plate; do not reuse
Rabbit Anti-Chicken IgY HRP conjugate (1000X)	-20°C, single use tube; 1/plate; do not reuse

- ELISA plate coated with rabbit anti-beta casein antibody, 2-8°C 3 days maximum.
- BSA for dilution/blocking buffer preparation: as recommended by vendor
- 10X TBS, 2-8°C, 1 week maximum
- Substrate TMBS, 2-8°C, as recommended by vendor
- Diluted detection antibody, HRP-conjugate and test samples: do not store, make just before use.

IX. Calculation of Results

(a) Manual Plate Reading:

(The relative $O.D._{450}$) = (the $O.D._{450}$ of each well) – (the $O.D._{450}$ of Zero well).

The standard curve can be plotted as the relative $O.D._{450}$ of each standard solution (Y) vs. the respective concentration of the standard solution (X). Known concentrations of the target protein are plotted on the X-axis and the corresponding $O.D._{450}$ on the Y-axis. The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding $O.D._{450}$. The greater the concentration of target protein in the sample, the higher the $O.D._{450}$.

Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

Determine concentration of target protein in unknown sample:

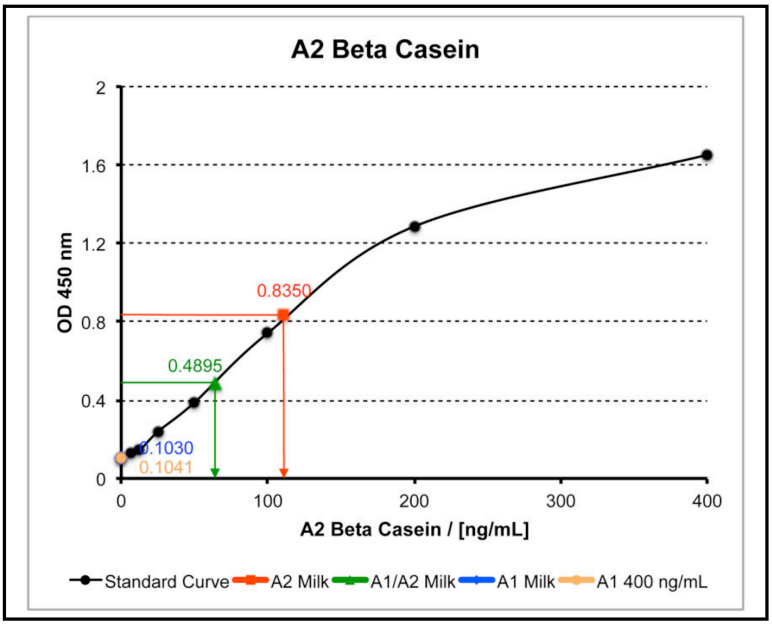
The target protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of antibody in the unknown sample. Determine the A2 concentration for the tested samples based on their OD450 and standard curve. Make sure that the final concentration is obtained by multiplying with dilution factor. Any OD450 reading is less than the OD450 reading from 6.25 ng/mL will need to be re-tested at a lower sample dilution. For instance, if you dilute your milk sample at 1:40,000 and its OD450 reading is 0.302 which is less than 0.332 from 25 ng/ml standard OD450 reading, you will need to dilute the milk sample at 1:4,000 or less in re-test.

PC Interface Plate Reading:
Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit; avoid using linear regression analysis. Perform a 4-PL regression analysis to calculate the concentration of target analyte in the samples. Multiply the result by the sample dilution factor. See Biosensis Technical note on ELISA data analysis for additional guidance on 4-PL curve fitting.

X. Typical Standard Curve (For Reference Only)

A2 Std (ng/mL)	OD1	OD2	Average OD
400	1.6584	1.6536	1.6560
200	1.2840	1.2903	1.2872
100	0.7368	0.7476	0.7422
50	0.4068	0.3688	0.3878
25	0.2615	0.2158	0.2387
12.5	0.1453	0.1425	0.1439
6.25	0.1338	0.1232	0.1285
0	0.1022	0.1055	0.10385
Sample	**OD1**	**OD2**	**Average OD**
A1 400 ng/ml	0.1046	0.1035	0.1041
A1 Milk 1:20,000	0.0984	0.1075	0.1030
A2 Milk 1:20,000	0.8478	0.8221	0.8350
A1/A2 heterozygote Milk 1:10,000	0.4906	0.4884	0.4895

Room temperature, 6 minutes



Graph demonstrates the dynamic range and high specificity of the beta Casein A2 ELISA. Note that samples containing A1 allele of beta casein fail to generate a signal even at 400 ng/mL concentrations (orange data point). Heterozygote animals (green data point) generate signals ~ 1/2 of those of homozygote animals (red data point) demonstrating the expected quantification of signal from such animals.