biosensis® Bovine A2 Beta Casein ELISA Kit Protocol

Catalog No: BEK-2365-1P

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

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I. Experimental Overview:

The Biosensis Bovine A2 Beta Casein ELISA Kit 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 pAbs are pre-coated on ELISA plate. A2 beta casein standards (6.25 ng/mL-400 ng/mL) and 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 the 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 sample need to be diluted to 1:10,000 to 1:50,000 since A2 level is high. For testing A2 contamination in A1 milk, sample can be diluted to 1:10-1:500 or greater.

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.

II. Materials Provided

Kit Components	Unit Size/Volume	Qty in Kit
ELISA Plate coated with rabbit anti-beta Casein pAbs	96 well	1
A2 beta casein standard (40 µg/mL (40 ng/µL)	20 µL	1
Chicken Anti-A2 beta casein specific pAbs, (0.3 mg/mL) (300X)	70 µL	1
Rabbit Anti-Chicken IgY HRP conjugate (1000X)	20 µL	1
BSA for Ab dilution buffer in 125 mL bottle	1 g	1
10X TBS in 125 mL bottle	100 mL	1
Tween-20 (Polyoxyethylene-Sorbitan Monolaurate), 50% solution, 1 mL tube	1 mL	1
TMBS Substrate in 30 mL bottle	6 mL	1
ELISA Record Sheet and insert instructions	--	1

WARNING: DO NOT MIX REAGENTS SUCH AS ANTIBODY BUFFERS ETC BETWEEN DIFFERENT ASSAYS IF USING MULTIPLE KITS. USE ONLY THE BUFFERS, STANDARDS & SOLUTIONS INCLUDED FOR YOUR SPECIFIC LOT. MIXING SOLUTIONS FROM DIFFERENT TARGET KITS CAN LEAD TO POOR KIT PERFORMANCE

All components with -20°C or 4°C are shipped on blue ice packs and will arrive cold or room temperature. See page 4 storage instructions for correct storage of kit and its components upon receipt.

III. Equipment 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. HCl, 1M for stopping reaction in final step

IV. Technical Hints

1. Spin all kit 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 kit performance will be poor

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V. Storage of Kit Components:

The unopened kit can be stored at 4°C for up to one 1 week if the kit will be used within that week. **If the kit 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 kit is 6 months if stored as directed below. Once opened, use all components within 1 week. Place components in appropriate temperature locations as directed below. ***Avoid repeated freeze thaw cycles on frozen items.***

ELISA Plate coated with rabbit Anti-beta Casein pAbs, 4°C
A2 beta casein standard, 40 µg/mL (40 ng/µL), -20°C
Chicken Anti-A2 beta casein specific pAbs, 0.3 mg/mL, -20°C
Rabbit Anti-Chicken IgY HRP conjugate, use at 1:1,000 dilution, -20°C
BSA for Ab dilution buffer in 125 mL bottle (need to add 100 mL TBST) 4°C
10X TBS, 100 mL, 4°C
Tween-20 (Polyoxyethylene-Sorbitan Monolaurate), 50%, 1 mL, 4°C
Substrate TMBS, 4°C
ELISA Record Sheet RT

VI. Reagent Preparations

Prepare TBS/TBST/solutions:

1. Prepare TBS: add 100 mL 10X TBS into 900 mL D.I. water, mix well; store at RT; filter through a 0.45 µm filter unit for lowest backgrounds.

2. Prepare TBST: Add 1 mL of the Tween-20 solution into the 1X TBS from step 1, and mix well, TBST will be used for preparing the antibody dilution buffer and for plate washing.

Prepare Antibody/Sample Dilution Buffer:

Add 100 mL TBST solution into the 125 mL BSA bottle and mix well. Do not over mix.

Preparation of Standards:

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

(b) 200 ng - 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) 12.5 ng/mL, and (tube #7) 6.25 ng/mL respectively. Aliquot 0.5 mL of antibody/sample buffer

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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 2st tube into 3nd 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. Store as directed below. Use sterile technique.

Note: Standard solution should be prepared no more than 2 hours prior to the experiment. The working standard solution may be stored at 4°C for up to 3 hours. Avoid repeated freeze-thaw cycles.

Preparation of Detection Antibody:

Prepare (10 mL per plate) A2 specific detecting pAb (Chicken IgY) in Ab dilution buffer at ~ 1:300 dilution, ie, add 35 μL A2 Detecting Abs into 10 ml Ab dilution buffer just before use. Do not store. See procedural instructions.

Preparation of Anti-Chicken HRP Secondary:

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 μL HRP conjugate into 10 mL Ab dilution buffer, mix well) just prior to use. Do not store. See procedural instructions.

VII. Sample Preparation and Dilution

Preparation of Samples:

Prepare the tested milk samples by diluting milk in Ab dilution buffer accordingly. In general, house milk sample need to be diluted to 1:10,000 to 1:50,000 since A2 level is high. Note dilutions can be as high as 1:100,000 for some samples. For testing A2 contamination in A1 milk, sample can be diluted to 1:10-1:500 or greater. Samples can be stored at 4°C for up to 3 hours; do not freeze.

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VIII. Assay Procedure

We recommend that standard solution dilutions and each sample are plated in duplicate. Read entire protocol before beginning. Prepare all buffer solutions ahead of time as directed except for the standards, actual samples, and secondary antibody dilutions. These should be made just before use.

1. Remove ELISA plate from storage and bring to room temperature. Remove the seal from ELISA plate, empty the solution and wash 3X with TBST, 1 minute each. Then add 100 µL/well of standard or above prepared sample in duplicate and make sure to record each well location on the ELISA Record Sheet

2. Mix the plate on an ELISA Plate shaker at 400 rpm for 2 hour at RT.

3. Wash 3X with TBST, 3 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 µL A2 Detecting Abs into 10 mL Ab dilution buffer.

5. Add the prepared A2 detecting pAb at 100 µL/well to the plate and shake at 400 rpm for 1 hour at RT.

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 µL HRP conjugate into 10 mL Ab dilution buffer, mix well). Add 100 µL/well to the plate.

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

9. Wash 5X with TBST, 3-5 minutes each.

10. Add 50 µL/well substrate (TMBS). Let the color to develop 5-10 minutes. Note the order and arrangement of the substrate addition. One must add the stop solution in the same order for best results. Color development will be quick.

11. Stop the reaction with 50 µl 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.

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

12. 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.

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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.

(b) 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.

X: Typical Standard Curve (for reference only)

Room temperature, 6 minutes

A2 Detecting Abs: 1:300

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

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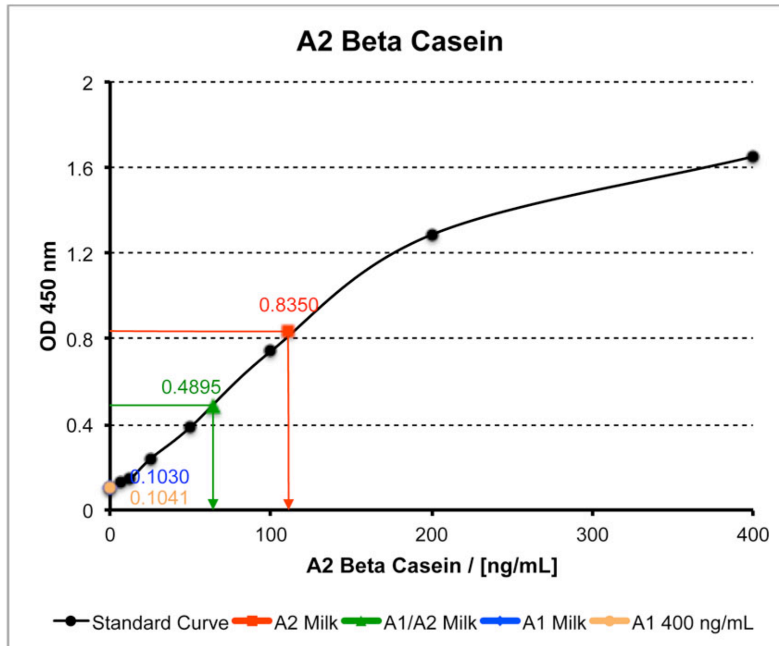
Sample	OD1	OD2	Average OD
A1 400 ng/ml	0.1046	0.1035	0.1041
A1 Milk 1:20,000	0.0984	0.1075	0.1030
A2 Milk 1:20,000	0.8478	0.8221	0.8350
A1/A2 heterozygote Milk 1:10,000	0.4906	0.4884	0.4895



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.

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