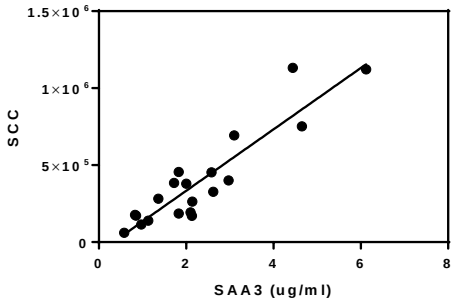
INTRODUCTION

SAA3, is the isoform of serum amyloid A that is expressed in bovine mammary epithelial cells and secreted into milk. It is a positive acute phase protein of ≈12 kDa. Levels increase because of inflammation and infection associated with mastitis. In studies at Veterinary Biomarkers Inc., we find that milk SAA3 levels correlate with somatic cell count (SCC).



The assay uses two cow SAA3 monoclonal antibodies: SAA3-11-11H3 for solid phase immobilization (microtiter wells) and HRP conjugated SAA3-11-5H6 for detection. Diluted milk samples and standards are incubated in microtiter wells together with HRP conjugate for one hour. If present, SAA3 molecules are sandwiched between the immobilization and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If SAA3 is present, a blue color develops. Color development is stopped by addition of Stop solution; changing the color to yellow. Absorbance is measured at 450 nm. The concentration of SAA3 is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-SAA3 coated plate (12 x 8-well strips)
- HRP conjugate: 11 ml
- SAA3 stock Store ≤ -20°C
- 20x Wash solution: TBS50-20, 50 ml
- Diluent: CSD50-1, 2 x 50 ml
- TMB: TMB11-1, 11 ml
- Stop solution: SS11-1, 11 ml

Materials required but not provided:

- Pipettors and tips
- Distilled or deionized water
- Polypropylene tubes
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm
- Graphing software

STORAGE

The SAA3 stock should be stored at or below -20°C. The remainder of the kit should be stored at 2-8°C. The microtiter plate should be kept in a sealed bag with desiccant. Kits will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature before use.
2. Reliable and reproducible results will be obtained when the assay is carried out with a complete understanding of the instructions and with adherence to good laboratory practice.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
4. Laboratory temperature will influence absorbance readings. Our ELISA kits are calibrated using shaking incubators set at 150 rpm and 25°C. Performance of the assay at lower temperatures will result in lower absorbance values.

WASH SOLUTION

The wash solution is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water. Unused wash buffer may be stored at 2-8°C for one week.

DILUENT

The diluent is specially formulated for measurement of SAA3 in cow milk. It is provided ready to use. Do not substitute other buffers.

STANDARD

1. The stock consists of 3 μg/ml of pure SAA3 in a stabilizing matrix. Prepare the 30 ng/ml standard by mixing 10.0 μl of stock with 0.99 ml of diluent.
2. Label four polypropylene tubes as 15, 7.5, 3.75 and 1.875 ng/ml. Dispense 0.5 ml of diluent into each.
3. Pipette 0.5 ml of the 30 ng/ml SAA3 standard into the tube labeled 15 ng/ml and mix. This provides the 15 ng/ml SAA3 standard.
4. Similarly prepare the remaining standards by two-fold serial dilution.

Use the standards within 30 minutes. Although the SAA3 stock is stable for several days at room temperature it should be frozen for optimum stability.

SAMPLES

This kit was designed specifically for measurement of SAA3 in milk. In milk with SCC in the range of 0.06 to 1.1 x 10^6 we found SAA3 levels ranging from 0.5 to 6 μg/ml. Levels as high as 40 μg/ml were found in milk from cows with severe mastitis. We suggest testing milk of normal appearance at a dilution of 400-fold, but optimum dilutions should be determined empirically. To avoid matrix effects, do not test milk at dilutions less than 100-fold. A 400-fold dilution can be obtained as follows.

1. Dispense 90 μl and 487.5 μl of diluent into separate microcentrifuge tubes.
2. Mix 10 μl of milk with 90 μl of diluent in the first tube. This represents a 10-fold dilution.
3. Prepare a 400-fold dilution of the sample by mixing 12.5 μl of the 10-fold diluted sample with 487.5 μl of diluent in the second tube.

PROCEDURE

1. Secure the desired number of 8-well strips in the cassette. Unused strips should be stored in a sealed bag with desiccant at 2-8°C.
2. Dispense 100 μl of standards and samples into the wells (we recommend that standards and samples be run in duplicate).
3. Add 100 μl of HRP-conjugate to each well.
4. Incubate on a plate shaker at 150 rpm and 25°C for one hour.
5. Empty and wash the microtiter wells 5x with 1x wash solution using a plate washer (400 μl/well).
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Dispense 100 μl of TMB into each well.
8. Incubate on an orbital micro-plate shaker at 150 rpm at 25°C for 20 minutes.
9. After 20-minutes, stop the reaction by adding 100 μl of Stop solution to each well.
10. Gently mix. It is important to make sure that all the blue color changes to yellow.
11. Read absorbance at 450 nm with a plate reader within 5 minutes.

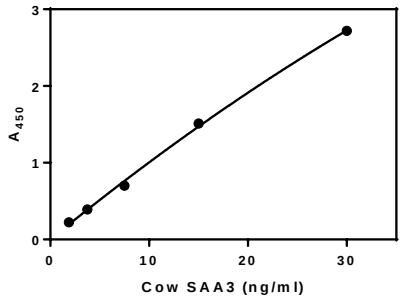
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus SAA3 concentration.
2. Fit the standard curve using graphing software. We typically fit to a single site binding (hyperbola) model.
3. Multiply the derived concentration by the dilution factor to determine the actual concentration in the milk sample.
4. If the A_{450} values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with absorbance at 450 nm on the Y-axis against SAA3 concentrations on the X-axis is shown below. This curve is for illustration only.

SAA3 (ng/ml)	A_{450}
30	2.719
15	1.511
7.5	0.702
3.75	0.390
1.875	0.223



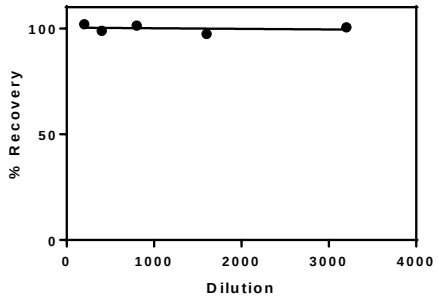
PERFORMANCE

Inter-Assay Precision (Precision between assays): Three samples of known concentration were tested in separate assays to assess inter-assay precision

Inter-Assay Precision			
Sample	1	2	3
n	3	3	3
Average (μg/ml)	0.111	6.43	30.39
Std. Deviation	0.005	0.54	0.75
CV%	4.7	8.4	2.5

Veterinary Biomarkers, Inc.,
124 Turner Lane, West Chester, PA 19380
484-200-9651 - info@vetbiomarkers.com – www.vetbiomarkers.com

Linearity: To assess the linearity of the assay, a milk sample containing SAA at a concentration of 6.1 μg/ml was serially diluted with diluent CSD50-1 to produce values within the dynamic range of the assay.



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For technical assistance please email us at info@vetbiomarkers.com

Veterinary Biomarkers, Inc.,
124 Turner Lane, West Chester, PA 19380
484-200-9651 - info@vetbiomarkers.com – www.vetbiomarkers.com