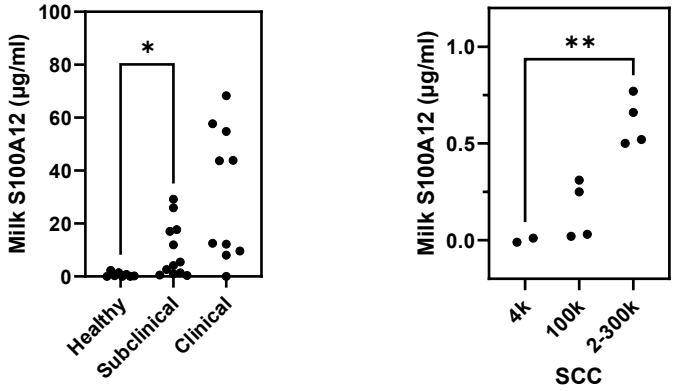
INTRODUCTION

S100A12 is a calcium and zinc binding protein that is released from neutrophils. It is a positive acute phase protein; levels increase during inflammatory diseases. Studies by Zhong et. al., showed that S100A12 mRNA levels were increased in milk from cows with subclinical mastitis (ref 1). In studies at Life Diagnostics, we found that S100A12 protein levels are significantly increased in milk from cows with subclinical and clinical mastitis. We also found that S100A12 levels correlated with milk somatic cell counts.



PRINCIPLE OF THE ASSAY

The assay uses antibodies generated against recombinant cow S100A12. Unconjugated antibody is used for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated antibody is used for detection. Standards and diluted samples are incubated in the microtiter wells for 45 minutes. The wells are subsequently washed. HRP conjugate is added and incubated for 45 minutes. This results in S100A12 molecules being sandwiched between immobilization and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If S100A12 is present, a blue color develops. Color development is stopped by the addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of S100A12 is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- S100A12 antibody coated 96-well plate (12 x 8-well strips)
- HRP Conjugate stock, 1 vial
- S100A12 stock, 1 vial, **store at -20°C**
- 20x Wash solution: CRPW50-20, 50 ml
- Diluent: CSDCA50-1, 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 or 96-well polystyrene plates
- 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 S100A12 stock must be stored at -20°C (do not store at lower temperatures). The remainder of the kit should be stored at 4°C and 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 except the S100A12 stock should be allowed to reach room temperature before use.
2. Reliable and reproducible results will be obtained when the assay is conducted with a complete understanding of the instructions and with adherence to good laboratory practice.
3. It is important that standards and samples be added to the ELISA plate quickly. If testing large numbers of samples, rather than pipetting standards and samples from individual tubes into the ELISA plate, we recommend the following: pipette an excess volume of standards and samples into wells of a blank polystyrene 96-well plate[1]. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 µl aliquots to the wells of the antibody-coated plate.
4. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
5. Laboratory temperature will influence absorbance readings. The assay was calibrated using a shaking incubator set at 150 rpm and 25°C. Performing the assay at lower temperatures and mixing speeds may result in lower absorbance values.

[1] Standards and sample dilutions may also be prepared directly in a blank polystyrene plate.

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 4°C for one week.

DILUENT

The diluent is ready to use. Allow it to warm to room temperature before use. DO NOT substitute other buffers

STANDARDS

1. The S100A12 stock is provided in a liquid format that must be stored at -20°C.
2. Label 8 polypropylene or glass tubes as 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0 ng/ml.
3. Using the tube labelled 10 ng/ml, prepare the 10 ng/ml standard as described on the stock vial label.
4. Dispense 250 µl of CSDCA50-1 diluent into the remaining tubes.
5. Prepare the 5 ng/ml standard by diluting and mixing 250 µl of the 10 ng/ml standard with 250 µl of diluent in the tube labeled 5 ng/ml.
6. Similarly prepare the 2.5 – 0.156 standards by two-fold serial dilution.

HRP CONJUGATE

The HRP conjugate is provided as a concentrated stock. Prepare the working conjugate by diluting with diluent CSDCA50-1 as described on the stock vial label.

SAMPLES

Suggested dilutions, based on our experience, are detailed below. The diluent provided with the kit (CSDCA50-1) must be used for dilution. DO NOT substitute other buffers.

Serum and Plasma: We found S100A12 levels up to 700 ng/ml in serum. We suggest that samples be evaluated at a dilution of 100-fold to obtain values within range of the standard. A 100-fold dilution can be obtained by mixing 2.0 ul of serum or plasma with 398 ul of diluent.

Milk: We found levels ranging from undetectable to 100 µg/ml in milk, depending on the source and disease status. Users must therefore determine optimal dilutions for their samples. That said, we found that a dilution of 5000-fold worked well for most samples we tested. A 5000-fold dilution can be obtained as follows.

1. Dispense 198 µl and 245 µl of diluent into separate microcentrifuge tubes, or wells of a 96-well plate.
2. Mix 2.0 µl of milk with 198 µl of diluent in the first tube. This gives a 100-fold dilution.
3. Mix 5 µl of the 100-fold diluted sample with 245 µl of diluent in the second tube. This gives a 5000-fold dilution.

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 4°C.
2. Dispense 100 µl of standards and samples into appropriate wells. We recommend that standards and samples be tested in duplicate.
3. Incubate on a plate shaker set at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 µl/well). If necessary, strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
5. Dispense 100 µl of HRP conjugate into each well.
6. Incubate on a plate shaker set at 150 rpm and 25°C for 45 minutes.
7. Empty and wash the microtiter wells as described in step 4.
8. Dispense 100 µl of TMB into each well.
9. Incubate on an orbital micro-plate shaker at 150 rpm and 25°C for 20 minutes.
10. After 20 minutes stop the reaction by adding 100 µl of Stop Solution to each well.
11. Gently mix. It is important to make sure that all the blue color changes to yellow.
12. Read absorbance at 450 nm[2] with a plate reader within 5 minutes.

RESULTS

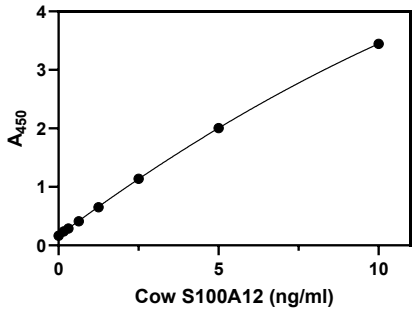
1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the S100A12 concentration. We suggest using a second order polynomial (quadratic) equation.
2. Derive the concentration of S100A12 in the samples.
3. Multiply the derived concentration by the dilution factor to determine the concentration in the sample.
4. If the absorbance values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

[2] If absorbance of the high standard is ≥4 when measured at 450 nm, absorbance of all standards and samples should be read at 405 nm.

TYPICAL STANDARD CURVE

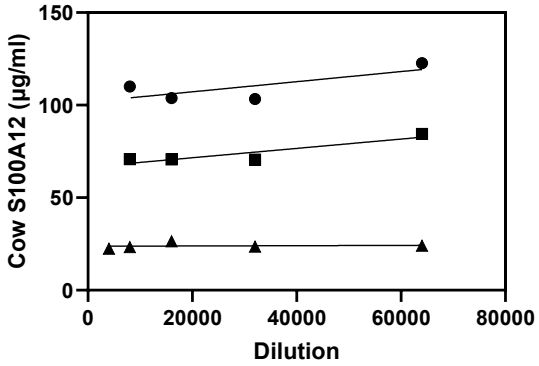
A typical standard curve is shown below. This curve is for illustration only.

S100A12 (ng/ml)	A_{450}
10	3.446
5	2.005
2.5	1.139
1.25	0.653
0.625	0.410
0.313	0.289
0.156	0.239
0	0.164



PERFORMANCE

Linearity: To assess the linearity of the assay, three milk samples with S100A12 concentrations of 24, 74, and 114 μg/ml were serially diluted to give values within range of the assay.



REFERENCES

1. Zhong K. et al. S100 calcium-binding protein A12 as a diagnostic index for subclinical mastitis in cows. Reprod Domest Anim . 2018 Dec;53(6):1442-1447. doi: 10.1111/rda.13273. Epub 2018 Jul 31.

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