INTRODUCTION

S100A12 is a calcium and zinc binding protein, released from neutrophils. It is a positive acute phase protein; levels increase during inflammatory diseases. It shows promise as a fecal biomarker of enteritis in dogs (ref 1).

PRINCIPLE OF THE ASSAY

The assay uses antibodies generated against recombinant dog 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: SB50-1, 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 S100A2 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.

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 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, and 0 ng/ml.
3. Using the tube labelled 5 ng/ml, prepare the 5 ng/ml standard as described on the stock vial label.
4. Dispense 250 µl of SB50-1 diluent into the remaining tubes.
5. Prepare the 2.5 ng/ml standard by diluting and mixing 250 µl of the 5 ng/ml standard with 250 µl of diluent in the tube labeled 2.5 ng/ml.
6. Similarly prepare the 1.25 – 0.078 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 SB50-1 as described on the stock vial label.

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

SAMPLES

Suggested dilutions, based on our experience, are detailed below.

Serum and Plasma: We found S100A12 levels up to 600 ng/ml in serum. We suggest that samples be evaluated at a dilution of 200-fold to obtain values within range of the standard. A 200-fold dilution can be obtained by mixing 2.0 ul of serum or plasma with 398 ul of diluent.

Fecal extracts: We prepared fecal extracts by mixing ~100 mg of feces with 0.5 ml of TBS and 0.1 ml of 1.6 mm stainless steel beads. The mixtures were homogenized in a Bullet Blender for 1 minute at setting 12. The homogenates were then centrifuged for 4 minutes at 15,000 rpm and the supernatants saved for testing. We found S100A12 levels up to 100 μg/ml. A dilution of 5000-fold worked well for most samples. 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 μl of serum 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.

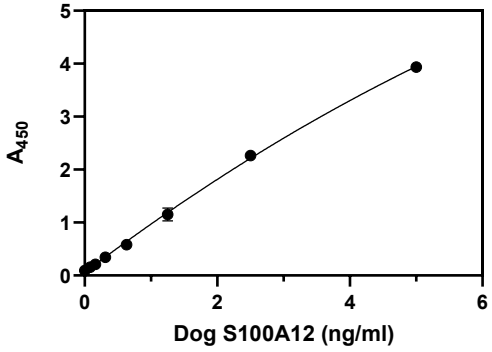
RESULTS

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 SAA 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.

TYPICAL STANDARD CURVE

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

S100A12 (ng/ml)	A_{450}
5	3.936
2.5	2.265
1.25	1.152
0.625	0.581
0.313	0.343
0.156	0.210
0.078	0.155
0	0.094



[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.

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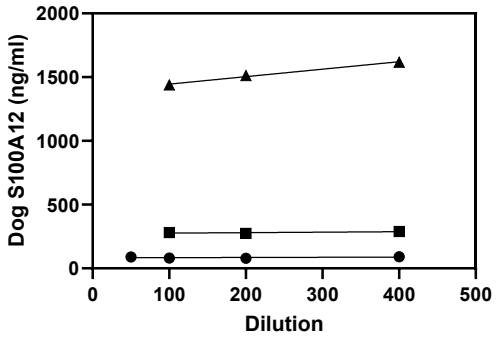
PERFORMANCE

Linearity: To assess the linearity of the assay, three serum samples with S100A12 concentrations of 90, 290, and 1478 ng/ml were serially diluted to give values within range of the assay.



REFERENCES

1. Heilmann RM. et al. Fecal S100A12 concentration predicts a lack of response to treatment in dogs affected with chronic eneropathy. Vet J. 2016 Sep:215:96-100 DOI: 10.1016/j.tvjl.2016.03.001. Epub 2016 Mar 7

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