DOG SERUM AMYLOID A (SAA) SPARCL™ ASSAY
Life Diagnostics, Inc., Catalog Number: SAA-SP-4

INTRODUCTION

Serum amyloid A (SAA) is a positive acute phase protein of ~12 kDa that is expressed in the liver and circulates in blood. It is a useful biomarker of inflammation and disease in dogs (refs 1-2). Serum levels can increase >100-fold during the acute phase response. In studies at Life Diagnostics, Inc., using this kit, we found SAA levels <200 ng/ml in serum from healthy dogs. Levels of 12.8±14.5 μg/ml (mean±SD, n=6) were found in serum from sick dogs. A level of 2.05 mg/ml was found in one sample.

PRINCIPLE OF THE ASSAY

The dog SAA SPARCL™[1] (Spatial Proximity Analyte Reagent Capture Luminescence, ref 3) assay uses two different peptide specific dog SAA antibodies. One is conjugated to horseradish peroxidase (HRP), the other is conjugated to acridan, a chemiluminescent substrate. When the HRP and acridan conjugated antibodies bind to SAA they are brought into close proximity. With the addition of hydrogen peroxide, HRP catalyzes oxidation of proximal acridan molecules causing a flash of chemiluminescence. Acridan conjugated antibodies distant from HRP produce no signal. This principle allows the development of a homogeneous assay that allows rapid measurement of SAA concentrations.

Serum samples are first treated with reagent alcohol in order to dissociate SAA from lipoproteins. Diluted samples and standards are then mixed with the HRP and acridan-conjugated antibodies in the wells of the 96-well SPARCL™ plate[2] provided with the kit. After incubation for 30 minutes on a shaker at 25°C and 150 rpm, the plate is placed into a luminometer. Trigger solution containing hydrogen peroxide is injected into each well and luminescence is immediately measured. The concentration of SAA is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-dog SAA HRP conjugate. **Store ≤ -70°C**
- Anti-dog SAA acridan conjugate. **Store ≤ -70°C**
- Dog SAA stock (3 vials). **Store ≤ -70°C**
- Diluent (CSD50-1), 2 x 50 ml
- Trigger solution, 7 ml
- White SPARCL™ plate (12 x 8-well)
- Clear untreated 96-well plate

Materials required but not provided:

- Reagent alcohol[3]
- Precision pipettes and tips
- Polypropylene tubes
- Vortex mixer
- Micro-Plate incubator/shaker
- Luminometer capable of simultaneous injection & measurement
- PC graphing software

STORAGE

Store the HRP conjugate, acridan conjugate and SAA stock at -70°C (they may be stored at -20°C for one week). The remainder of the kit should be stored at 2-8°C. The SPARCL™ plate should be kept in a sealed bag with desiccant and antioxidant. The kit will remain stable for at least six months from the date of purchase, provided that the components are stored as described.

GENERAL INSTRUCTIONS

The dilution buffer and 8-well strips used in the assay should be allowed to reach room temperature (25°C) before use.

STANDARD PREPARATION

The dog SAA stock is comprised of lyophilized dog SAA in a carrier protein matrix. The SAA content was determined by reference to purified dog SAA prepared at Life Diagnostics, Inc.

1. Reconstitute the lyophilized dog SAA stock with diluent as described on the vial label. Mix gently until dissolved. The concentration of SAA in the reconstituted stock is indicated on the label.
2. Label 6 polypropylene tubes[4] as 20, 10, 5, 2.5, 1.25 and 0.625 ng/ml.
3. Into the tube labeled 20 ng/ml, pipette the volume of diluent detailed on the SAA stock vial label. Then add the indicated volume of SAA stock and mix gently. This provides the 20 ng/ml standard.
4. Dispense 150 μl of diluent into the tubes labeled 10, 5, 2.5, 1.25 and 0.625 ng/ml.
5. Pipette 150 μl of the 20 ng/ml SAA standard into the tube labeled 10 ng/ml and mix. This provides the 10 ng/ml SAA standard.
6. Similarly prepare the remaining standards by serial dilution.

Please Note: Use the standards within one hour of preparation.

SAMPLE PREPARATION

Prior to testing, serum samples must be treated with reagent alcohol in order to dissociate SAA from lipoproteins which interfere with SAA measurement. Mix three volumes of each serum sample (45 μl) with one volume (15 μl) of reagent alcohol. We recommend using either a positive displacement pipette or a Hamilton syringe to accurately dispense the reagent alcohol. Please note that after addition of alcohol the original sample is diluted 1.33 fold. The alcohol treated serum samples should be used within 15 minutes of preparation.

The alcohol treated samples must be further diluted at least 100-fold in order to avoid matrix effects. This can be achieved by mixing 2.50 μl of alcohol treated serum with 247.5 μl of diluent. Please note that this represents a 133.3-fold dilution of the original sample. If further dilution is needed, use diluent CSD50-1. Do not use other diluents.

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[1] The SPARCL technology was developed by Lumigen Corp.

[2] The plate provided with the kit has been treated with a reagent that reduces background chemiluminescence. Untreated plates cannot be used.

[3] Reagent Alcohol consists of ethanol, isopropyl alcohol and methanol in the ratio 90:5:5.

[4] Dilutions of standards can be performed in wells A1-A8 of the clear untreated 96-well plate provided with the kit. This allows rapid transfer of standards to the white SPARCL™ plate using a multipipettor. Diluted samples can also be first aliquoted into appropriate wells of the clear polystyrene plate and subsequently transferred to the SPARCL™ plate with a multipipettor. If using this method, ensure that an excess volume is aliquoted into the clear plate in order to ensure complete transfer of 50 μl aliquots to the SPARCL™ plate.

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The dog SAA SPARCL™ assay uses a homogeneous format and is therefore susceptible to a prozone or "hook effect" at high SAA concentrations. Samples should ideally be tested at several dilutions in order to eliminate false low values. At a minimum, we suggest testing each sample at dilutions of 133.3 and 4000-fold.

1. The 133.3-fold dilution is prepared as described above.
2. A 4000-fold dilution can be prepared by mixing 6.67 µl of the 133.3-fold diluted sample with 193.33 µl of diluent.

Samples testing out of range at a 4000-fold dilution should be further diluted and re-tested.

CONJUGATE MIX PREPARATION

Instructions for preparation of the conjugate mix are detailed on the box that contains the HRP and acridan conjugates. If necessary, after thawing, briefly centrifuge to ensure that the contents are at the bottom of the tubes. Prepare the mix shortly before use using the diluent provided with the kit.

LUMINOMETER SETUP

1. The luminometer must be capable of injection and simultaneous measurement of luminescence without any delay.
2. Prime the luminometer injection port with 1 ml of trigger solution.
3. Place the injection needle into the injection port as needed for BMG luminometers.
4. Program the luminometer to inject 37.5 µl of trigger solution per well and to measure from time zero for 1 second (50 x 0.02 second intervals).
5. Define the format of the assay using the luminometer software.
6. Because the white SPARCL™ plate is provided as a 12 x 8-well strips, allowing use of fewer than 96-wells, make sure that the luminometer is programmed to inject trigger solution only into the wells being used.
7. We use a BMG LUMIstar Omega set at a gain of 3600. Optimal gain should be determined by the end user.
8. There are a number of manufacturers of luminometers that are equipped to run a SPARCL™ assay. Please contact Life Diagnostics or Lumigen (www.lumigen.com) to discuss your luminometer.

PROCEDURE

1. Before starting the assay ensure that the luminometer is primed with trigger solution and that the injection needle is positioned in the injection port.
2. Secure the desired number of SPARCL™ 8-well strips in the holder. Immediately seal unused strips in the resealable bag with desiccant and antioxidant. Store unused strips at 2-8°C.
3. Aliquot 25.0 µl of conjugate mix into each well.
4. Dispense 50.0 µl of standards and diluted samples into the wells (we recommend that standards and samples be tested in duplicate).
5. Incubate on an orbital micro-plate shaker at 150 rpm 25°C for 30 minutes.
6. After the 30-minute incubation, place the plate in the luminometer and measure luminescence after injection of trigger solution (37.5 µl).
7. Remove the plate from the luminometer and discard the used strips. Keep the plate frame if future use is intended.

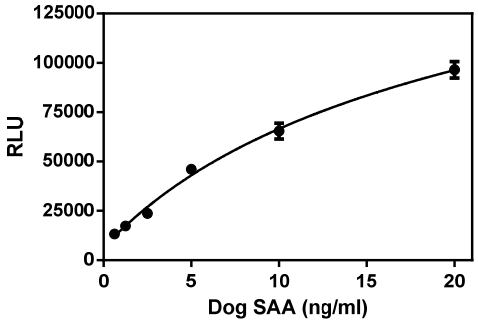
CALCULATION OF RESULTS

1. Before calculating results, review the raw data. If artefacts (RLU spikes) are apparent immediately after injection of trigger solution, eliminate that portion of the luminescence profile from analysis for all wells.
2. Using graphing software, construct a standard curve by plotting the luminescence (RLU) for the standards versus the SAA concentration in ngml.
3. Fit data using graphing software. We find that a single site, total and nonspecific binding model works well.
4. Derive the corresponding concentration of SAA in the diluted samples from the standard curve.
5. Multiply the derived concentration by the dilution factor to determine the actual concentration of SAA in the serum or plasma sample.
6. If the RLU values of diluted samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with RLU plotted on the Y-axis versus SAA concentrations on the X-axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns.

SAA(ng/ml)	RLU
20	96496
10	65456
5	46145
2.5	23728
1.25	17313
0.625	13244



REFERENCES

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2. Lowrie M, et al. The role of acute phase proteins in diagnosis and management of steroid-responsive meningitis arteritis in dogs. The Veterinary Journal. 182:125-130 (2009).
3. Akhavan-Tafti H. et al. A homogeneous chemiluminescent immunoassay method. J Am Chem Soc. 20;135(11):4191-4 (2013)

Rev 081216

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