

CAT SERUM AMYLOID A (SAA) SPARCL™ ASSAY

Life Diagnostics, Inc., Catalog Number: SAA-SP-8

INTRODUCTION

Serum amyloid A (SAA) is a positive acute phase protein of ~12 kDa that is expressed in the liver and circulates in blood. Levels can increase >50-fold in cats, making it a useful biomarker of inflammation and disease (refs 1 & 2).

PRINCIPLE OF THE ASSAY

The cat SAA SPARCL™¹ (Spatial Proximity Analyte Reagent Capture Luminescence, ref 3) assay uses two different cat SAA antibodies that were developed at Life Diagnostics, Inc. 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 or plasma samples are first heated at 60°C for two hours to dissociate SAA from lipoproteins that interfere with antibody binding to SAA (ref 4). Diluted samples and standards are then mixed with HRP and acridan-conjugated antibodies in the wells of the 96-well SPARCL™ plate² 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-cat SAA HRP conjugate **Store ≤ -70°C**
- Anti-cat SAA acridan conjugate **Store ≤ -70°C**
- Cat SAA stock **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:

- Dry-block heater or incubator capable of heating to 60°C
- Precision pipettes and tips
- Microcentrifuge tubes with sealable caps
- 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 cat SAA stock is comprised of pure cat SAA diluted in a stabilizing carrier protein matrix. Thaw the stock shortly before use.

1. Label 8 polypropylene tubes³ as 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 ng/ml.
2. Into the tube labeled 100 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 100 ng/ml standard.
3. Dispense 150 µl of diluent into the tubes labeled 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 ng/ml.
4. Pipette 150 µl of the 100 ng/ml SAA standard into the tube labeled 50 ng/ml and mix. This provides the 50 ng/ml SAA standard.
5. Similarly prepare the remaining standards by two-fold serial dilution.

The standard stock and working standards do not have to be heated at 60°C.

SAMPLE PREPARATION

Prior to testing, serum or plasma samples must be heated at 60°C for two hours to dissociate SAA from lipoproteins that interfere with its measurement.

1. Pipette 100 µl aliquots of serum or plasma into 0.5 ml microcentrifuge tubes and tightly seal the caps.
2. Place the tubes in either a dry block heater (with a heated lid) or an incubator pre-heated to 60°C.
3. After two hours remove the tubes and allow them to cool for a few minutes at room temperature.
4. Centrifuge the tubes briefly to remove any condensate from the cap and vortex to mix the contents.

After heating, it is necessary to further dilute the samples. In studies at Life Diagnostics, we found SAA levels ranging from <0.1 to >500 µg/ml. We suggest that each sample be tested at dilutions of 100, 1000 and 10,000-fold to increase the likelihood of obtaining values within range of the standard curve.

1. A 100-fold dilution can be prepared by mixing 2.0 µl⁴ of the heated sample with 198.0 µl of diluent.

¹The SPARCL technology was developed by Lumigen Corp.

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

³Dilutions 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.

⁴We found that ~10% of samples form a gel after heating. If this occurs, mix the sample by flicking the bottom of the tube. Slowly withdraw 2.0 µl of sample using a precision

- Mix 20 μl of the 100-fold diluted sample with 180 μl of diluent to obtain a 1000-fold dilution.
- Mix 20 μl of the 1000-fold diluted sample with 180 μl of diluent to obtain a 10,000-fold dilution.

Use the diluent provided with the kit (CSD50-1) for dilution. Do not substitute other buffers.

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

- The luminometer must be capable of injection and simultaneous measurement of luminescence without any delay.
- Prime the luminometer injection port with 1 ml of trigger solution.
- Place the injection needle into the injection port as needed for BMG luminometers.
- 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).
- Define the format of the assay using the luminometer software.
- 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.
- We use a BMG LUMIstar Omega set at a gain of 3600. Optimal gain should be determined by the end user.
- 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

- Before starting the assay ensure that the luminometer is primed with trigger solution and that the injection needle is positioned in the injection port.
- 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.
- Aliquot 25.0 μl of conjugate mix into each well.
- Dispense 50.0 μl of standards and diluted samples into the wells (we strongly recommend that standards and samples be tested in duplicate).
- Incubate on an orbital micro-plate shaker at 150 rpm 25°C for 30 minutes.
- After the 30-minute incubation, place the plate in the luminometer and measure luminescence after injection of trigger solution (37.5 μl).
- Remove the plate from the luminometer and discard the used strips. Keep the plate frame if future use is intended.

CALCULATION OF RESULTS

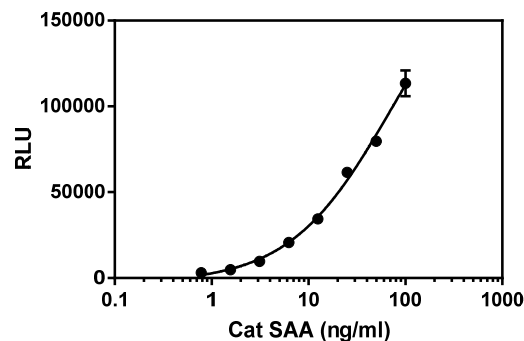
- Before calculating results, review the raw data. If artefacts (RLU spikes) are apparent immediately after injection of trigger solution, eliminate that portion of the luminescence profile from analysis for all wells.

- Using graphing software, construct a standard curve by plotting the luminescence (RLU) for the standards versus \log_{10} of the SAA concentration in ng/ml.
- Fit the data using a variable slope, four-parameter logistic curve ($x = \log_{10}$ concentration).
- Derive the concentration of SAA in the samples from the standard curve (i.e. determine the antilog).
- Multiply the derived concentration by the dilution factor to determine the actual concentration of SAA in the serum or plasma sample.
- 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 an example and should not be used to calculate unknowns.

SAA(ng/ml)	RLU
100	113466
50	79666
25	61554
12.5	34377
6.25	20674
3.13	9677
1.56	4858
0.78	3007



REFERENCES

- Tamamoto T, et al. Verification of measurement of the feline serum amyloid A (SAA) concentration by human SAA turbidimetric immunoassay and its clinical application. *J. Vet. Med. Sci.* 70(11):1247-1252 (2008)
- Tamamoto T, et al. Serum amyloid A as a prognostic marker in cats with various diseases. *J. Vet. Diag. Invest.* 25(3):428-432 (2013)
- Akhavan-Tafti H. et al. A homogeneous chemiluminescent immunoassay method. *J Am Chem Soc.* 20;135(11):4191-4 (2013)
- Benditt EP, Eriksen N and Hanson RH. Amyloid protein SAA is an apoprotein of mouse plasma high density lipoprotein. *Proc. Natl. Acad. Sci.* 76(8):4092-4096 (1979)

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pipettor. If necessary, cut the end off the pipette tip to better withdraw the viscous sample.