INTRODUCTION

Serum amyloid A (SAA) is a positive acute phase protein that is expressed in liver and circulates in blood. In cats it can increase >1000-fold during inflammation and infection.

PRINCIPLE OF THE ASSAY

The cat SAA assay uses SPARCL™[1] technology. Two SAA antibodies are used. One is conjugated to horseradish peroxidase (HRP), the other to acridan, a chemiluminescent substrate. When HRP and acridan conjugates bind to SAA they are brought into proximity. Upon addition of hydrogen peroxide, HRP catalyzes oxidation of proximal acridan molecules causing a flash of luminescence that is proportional to SAA concentration.
Diluted serum or plasma samples and standards are dispensed into test tubes and mixed with 0.5 ml of acridan and HRP conjugates. After 45 minutes, tubes are placed in the VetBio-1 luminometer. Luminescence is measured after injection of a background reducer, which eliminates nonspecific luminescence, followed by trigger-solution containing hydrogen peroxide. The concentration of SAA is automatically calculated from the ratio of sample luminescence to that of the standard. The assay sequence is illustrated below.

Equilibrate reagents to room temperature
⇩
Prime the luminometer
⇩
Program the luminometer and save the program
⇩
Dilute samples
⇩
Prepare standard
⇩
Add blank, standard and diluted samples to tubes (100 µl/tube)
⇩
Reconstitute the acridan/HRP conjugate mix (5.5 ml/vial)
⇩
Add conjugate mix to the tubes (0.5 ml/tube)
⇩
Incubate tubes at room temp for 45 min
⇩
Run the Quick Prime program
⇩
Load the program. Test standards and samples

KIT COMPONENTS

Materials provided with the kit:

- Acridan & HRP conjugates, 5 vials **Store at -20°C**
- SAA stock, 1 vial. **Store at -20°C**
- Diluent: CSD50-1, 2 x 50 ml
- Background Reducer: BR9-1, 9 ml
- Trigger Solution: TS12-1, 12 ml

Materials required but not provided:

- VetBio-1 luminometer
- Precision pipettors and tips
- 12 x 75 mm borosilicate tubes[2]
- 12 x 75 mm test tube racks
- De-ionized water
- 15-ml centrifuge tubes & rack
- Microcentrifuge tubes

STORAGE

Store the conjugate and SAA stock vials at or below -20°C. The remainder of the kit should be stored at 4°C.

GENERAL INSTRUCTIONS

1. Please take the time to completely read all instructions before starting your assay**.** Contact us if you need clarification.
2. All reagents used in the assay should be allowed to reach room temperature before use.
3. It is important that the assay is performed in an area free of UV-light (sunlight). Therefore, please close window blinds. UV-light can cause borosilicate glass tubes to phosphoresce, leading to interference in the assay.

[1] SPARCL technology, using acridan- and HRP-conjugated antibodies, was developed by, and is licensed from Lumigen Corp.
[2] Only cylindrical 12 x 75 mm borosilicate glass tubes can be used. Do not use polystyrene or polycarbonate tubes.

LUMINOMETER SETUP

1. Turn the VetBio-1 luminometer on.
2. Place the tubing from injector 1 into the tube containing Background Reducer. Tube holders are positioned adjacent to the injectors.
3. Place the tube from injector 2 into the tube containing Trigger Solution.
4. From the Protocol manager on the keypad select “Prime & Wash,” "Prime," then “Start.”
5. Open the luminometer drawer and insert an empty 12 x 75 mm tube.
6. Close the drawer and press “Start” again.
7. Wait for priming to complete. Open the drawer and discard the tube.
8. From the Protocol manager select “Measure” followed by “Cat SAA.”
9. Select “Edit.”
10. The experiment setup screen will be displayed. Enter the sample dilution, replicates for the blank/standard, replicates for the samples, then press “Start.”
11. Enter the number of samples to be evaluated.
12. Enter the sample IDs and dilution factor(s), if different than the default dilution. You can also enter experimental comments.
13. After entering information, press save. The experiment will be saved as a “prepared experiment.”

SAMPLE PREPARATION

This assay was designed for measurement of SAA in serum. We recommend testing at a 1000-fold dilution.

1. Dispense 198 µl and 225 µl of CSD50-1 diluent into separate tubes (or wells of a 96-well polystyrene plate).
2. Mix 2.0 µl of serum with 198 µl of diluent in the first tube/well. This gives a 100-fold dilution.
3. Mix 25 µl of the 100-fold diluted sample with 225 µl of diluent in the second tube/well. This gives a 1000-fold dilution.

STANDARD PREPARATION

The standard is provided in lyophilized form.

1. Reconstitute the stock with distilled or deionized water as described on the stock vial label.
2. Prepare the 25 ng/ml standard by diluting the reconstituted stock with CSD50-1 diluent as detailed on the stock vial label.

Freeze unused reconstituted stock at or below -20°C.

CONJUGATE MIX PREPARATION

1. The acridan and HRP conjugate mix should be prepared just before use.
2. Tap the vial to ensure that the contents are at the bottom of the vial before carefully removing the stopper.
3. Add 5.5 ml of diluent CSD50-1 to the vial. Insert the stopper and mix gently by inversion several times.
4. Each vial of reconstituted conjugate mix provides enough reagent to measure 0 and 25 ng/ml standards and up to eight samples.[3]

PROCEDURE

The following procedure assumes that standards and samples are tested as singlets. If using replicates, modify the procedure appropriately.

STEP 1

1. Determine the number of 12 x 75 mm borosilicate glass tubes required (one each for the blank, standard, and the samples). Ensure that all the tubes you intend to use fit easily in the VetBio-1 sample holder before beginning the test.
2. Pipet 100 µl of diluent into tube 1. This serves as the zero standard or blank.
3. Pipet 100 µl of the 25 ng/ml SAA standard into tube 2.
4. Pipet 100 µl of the diluted samples into tubes 3, 4, 5…
5. Add 0.50 ml of freshly prepared conjugate mix to each tube and mix gently. A vortex mixer may be used if available. Avoid creating bubbles.
6. Incubate the mixtures at room temperature for 45 minutes.

STEP 2

1. One to two minutes before the end of the incubation step, insert an empty tube into the sample holder. Select “Quick Prime” from the Protocol manager, close the drawer and press start. Discard the tube after completion. This step ensures that the injectors are fully loaded.
2. Recall your saved experiment and press start.
3. After the end of the incubation, insert tube 1 (Blank) into the sample holder of the luminometer and close the drawer. When the luminescence value is recorded on the screen open the drawer and discard the tube.
4. Similarly measure luminescence of the standard (tube 2), followed by the samples (tubes 3, 4, 5…).
5. SAA concentrations of the samples are automatically calculated.
6. After measurement of the last sample, select “End.”
7. Results will be saved and can be recalled. They may be exported as Excel or pdf files via a USB stick.

RESULTS

If RLU/s values for diluted samples are between the blank and standard, assume that the calculated values are correct. If RLU/s values exceed that of the standard, SAA concentrations may be underestimated. If an accurate measurement is required, such samples should be retested at a higher dilution.

[3] If using multiple vials of conjugates to measure more than eight samples, combine the reconstituted contents of all vials and mix briefly before dispensing 0.5 ml aliquots into the reaction tubes. Larger volumes of background reducer and trigger solution must also be used.

LUMINOMETER MAINTENANCE

The luminometer injectors must be cleaned with distilled or deionized water at the end of each day that the instrument is used to avoid clogging.

1. From the Protocol manager screen select “Prime & Wash.”
2. Select “Backprime” to return unused reagents to the respective tubes.
3. Cap the background reducer and trigger solution tubes and store them at 4°C.
4. Wipe the tubing from injectors 1 and 2 and place them in separate 15 ml centrifuge tubes containing distilled or deionized water.
5. Select “Wash” from the “Prime & Wash” screen. Press “Start,” insert an empty tube into the luminometer, close the drawer and press “Start.”
6. Discard the tube.
7. Insert an empty tube and repeat steps 5 and 6.
8. Leave the injector tubing immersed in water.
9. Switch the luminometer off. It should not be left powered-up overnight.

ASSAY PERFORMANCE

Typical data.

Results are shown below from an assay in which serum samples from three cats were tested at a 10000-fold dilution, as singlets.

Cat SAA Exp#002 18.12.2019 22:28:58

Single Assay	Start by :	DOOR	Blank :	ON
Measurement	Delay [s] :	2.600	Time [s] :	0.800
Injector 1	Delay [s] :	1.000	Volume [µL] :	50
Injector 2	Delay [s] :	2.000	Volume [µL] :	100

Experiment comment: Serum test

Sample	Dilution	Rep	RLU/s	Conc [µg/mL]
Blank			2,325	0
Standard			545,518	0.025
1	10000		114,059	51.425
2	10000		4,020	0.78011
3	10000		17,185	6.8392

Reproducibility. Three serum samples with different SAA levels were tested in triplicate (Table 1) or as singlets (Table 2) in three separate assays at dilutions of 100, 1000, or 10000-fold. Levels of SAA are reported as µg/ml (mean ± SD, in Table 1).

Table 1.

Sample	Dilution	Assay 1	Assay 2	Assay 3
1	10000	53.7±5.4	72.3±15.4	67.6±2.4
2	100	0.35±0.04	0.45±0.14	0.38±0.01
3	1000	4.82±0.02	5.99±1.12	5.57±0.17

Table 2.

Sample	Dilution	Assay 1	Assay 2	Assay 3
1	10000	55.5	56.4	66.8
2	100	0.32	0.61	0.37
3	1000	4.84	5.50	5.37

FREQUENTLY ASKED QUESTIONS

1. Can I dilute the standard and samples, then use them later?
   - No. Once diluted in the assay buffer, SAA has limited stability; after one hour, recovered values decrease by approximately 10%. Diluted standard and samples should be used within 10-minutes.
2. Can I reconstitute the acridan/HRP conjugate reagent and use it later?
   - No. When reconstituted, the conjugate mix has limited stability. Luminescence signals decrease by approximately 25% after one hour. The reconstituted conjugate mix should be used within 10-minutes.
3. How stable is SAA in serum?
   - We find that SAA is quite stable in serum, samples can be stored in tightly sealed vials for at least one day at 4°C and at least one year at -20°C.
4. How stable are the Background Reducer and Trigger Solution?
   - Both are stable at room temperature for several days. However, after use they should be stored in capped vials in the refrigerator. Be careful not to switch caps.

Rev 091622

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