HORSE SAA VETBIO-1 ASSAY

SAA-HORSE-VB

INTRODUCTION

Serum amyloid A (SAA) is a positive acute phase protein that is expressed in liver and circulates in blood. In horses it can increase >100-fold during inflammation and infection. We measure SAA levels of ~2 μg/ml (2 mg/L) in serum from healthy horses and levels up to 2500 μg/ml (2500 mg/L) in serum from sick horses.

The horse 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.

SAA binds to lipoproteins that interfere with its measurements. Samples are therefore mixed with dissociation buffer and then further diluted prior to measurement. Diluted samples and standards (100 μl) 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 background reducer (50 μl), that eliminates nonspecific luminescence, followed by trigger-solution containing hydrogen peroxide (100 μl). The concentration of SAA is automatically determined from the ratio of sample luminescence to that of the 100 ng/ml standard. The assay sequence is illustrated below.

Prime and program the VetBio-1 luminometer

⇩

Dissociate and dilute samples

⇩

Prepare standard

⇩

Add blank, standard and 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

⇩

Measure luminescence after 45 min

KIT COMPONENTS

Materials provided with the kit:

- Acridan & HRP conjugates, 5 — Store at -20°C
- SAA stock — Store at -20°C
- Dissociation buffer: DB30-1, 30 ml
- Diluent: CSD50-1, 2 x 50 ml
- Background reducer: BR9-1, 9 ml
- Trigger solution: TS12-1, 12 ml
- 15 ml centrifuge tubes, 2
- 5 ml centrifuge tubes, 2

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

STORAGE

Store the conjugate and SAA stock vials at or below -20°C. The remainder of the kit should be stored at 2-8°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.

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”.

[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.

5. Open the luminometer drawer and insert an empty 12 x 75 mm tube.
6. Close the drawer and click “Start” again.
7. Wait for priming to complete, open the drawer and discard the tube.
8. From the Protocol manager select “Measure” followed by “Horse SAA”.
9. Select “Start”.
10. The experiment setup screen will be displayed, allowing you to enter experiment name, comments, and the number of samples. After entering the information select “Start”.
11. Enter the sample IDs and dilution factor(s), if different than the default dilution factor.
12. The luminometer is now ready for use.
13. Press “Start” when you are ready to measure luminescence.

REPLICATES

The VetBio-1 luminometer protocols are configured to run singlets of the blank, standard and samples. At the discretion of the user it can be configured to run replicates.

14. From the protocol manager select “Measure” and the program you would like to modify.
15. Select “New” and “Copy Protocol”.
16. Increase the Replicates as desired.
17. Create a new protocol name.
18. Select “Protocols” and save the new protocol.

SAMPLE PREP

This assay was designed for measurement of SAA in serum or plasma. Samples are first dissociated by a 100-fold dilution in dissociation buffer followed. Dissociated samples are then diluted 100-fold in diluent CSD50-1 to give a final dilution of 10,000-fold. For each sample perform the following.

1. Mix 10.0 µl of sample with 0.99 ml of dissociation buffer DB1-30 (100-fold dilution).
2. Incubate at room temperature for at least 10-minutes (dissociated samples are stable for several hours at room temperature).
3. Mix 10.0 µl of the dissociated sample with 0.99 ml of diluent CSD50-1 (10,000-fold dilution).
4. Use the dissociated/diluted samples within 10 minutes.

STANDARD PREP

Prepare the 100 ng/ml standard by diluting 4.0 µl of the SAA stock with 3.996 ml of CSD50-1 diluent.

CONJUGATE MIX PREP

1. The acridan and HRP conjugate mix should be prepared just before use (step 5 in the Procedure section).
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 100 ng/ml standards and up to eight samples.[3]

PROCEDURE

1. Determine the number of 12 x 75 mm borosilicate glass tubes required for the assay. Ensure that all tubes fit easily in the VetBio-1 sample holder.
2. Pipet 100 µl of diluent into assay tube one. This serves as the zero standard.
3. Pipet 100 µl of the 100 ng/ml SAA standard into tube two.
4. Pipet 100 µl aliquots of the diluted samples into tubes 3, 4, 5… as defined by your assay format.
5. Add 0.50 ml of freshly prepared conjugate mix to each tube and mix gently. A vortex mixer may be used if available but is not necessary.
6. Incubate the mixtures at room temperature.
7. After 45 minutes insert tube 1 into the sample holder of the luminometer and close the drawer. The luminometer automatically injects background reducer and trigger solution, then measures luminescence (RLU/s).
8. Once the RLU/s value is recorded on the screen open the drawer and discard the tube.
9. Determine luminescence for the remaining tubes.
10. SAA concentrations are automatically calculated.
11. After measurement of the last sample, select “End”.
12. Results may be exported via a USB stick in Excel, HTML, or pdf format.

LUMINOMETER MAINTENANCE

The luminometer injectors must be cleaned with distilled or deionized water at the end of each day of use to avoid clogging of the injector ports.

1. From the Protocol manager screen select “Prime & Wash”.
2. Select “Backprime” to return unused reagents to the respective tubes.
3. If future use of the reagents is intended store the sealed tubes in a refrigerator.
4. Place the tubing from injectors 1 and 2 into 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”.

[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.

6. Discard the tube.
7. Leave the injector tubing immersed in water.
8. Switch the luminometer off. It should not be left on overnight.

ASSAY PERFORMANCE

Typical data.

Results are shown below from an assay in which serum samples from three horses with colic were tested at a 100-fold dilution, as singlets.

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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: Colic serum

Sample	Dilution	Rep	RLU/s	Conc [µg/mL]
Blank			2,304	0
Standard			259,792	0.1
19	100		183,012	7.0181
24	100		6,746	0.17253
28	100		17,511	0.59061

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

Table 1.

Sample	Final Dilution	Assay 1	Assay 2	Assay 3
F	2000	23.5±0.3	32.5±8.7	32.1±1.7
H	4000	193±4	276±69	168±50
K	40000	2531±791	2664±883	2284±395

Table 2.

Sample	Final Dilution	Assay 1	Assay 2	Assay 3
F	2000	23.2	22.4	33.8
H	4000	190	210	136.6
K	40000	2544	2427	1899

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 ~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 ~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 is the SAA stock?
   - The SAA stock is stable for at least 10 days at room temperature and at least one year at -20°C.
5. 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.

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