INTRODUCTION

CRP (C-reactive protein) levels increase in dog serum because of injury, infection and cancer. CRP can therefore be used to diagnose and monitor disease.

PRINCIPLE OF THE ASSAY

The dog CRP assay uses SPARCL™[1] technology. Two CRP antibody conjugates are used; one to horseradish peroxidase (HRP), the other to acridan, a chemiluminescent substrate. When HRP and acridan conjugates bind to CRP 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 CRP concentration.

Diluted serum or plasma samples and standards (100 µl) are dispensed into test tubes and mixed with 0.5 ml of combined 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 CRP is determined from the ratio of sample luminescence to the that of the 10 ng/ml standard.

Prime and program the VetBio-1 luminometer

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Dilute samples

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Prepare 10 ng/ml standard

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Add blank, standard and samples to tubes (100 µl/tube)

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Reconstitute the acridan/HRP conjugate mix (5.5 ml/vial)

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Add conjugate mix to the tubes (0.5 ml/tube)

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Incubate tubes at room temp for 45 min

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Measure luminescence

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Acridan & HRP conjugates, 5 — Store at -20°C
- Dog CRP standard, 5 — Store at -20°C
- Diluent: CSD50-1, 2 x 50 ml
- Background reducer: BR9-1, 9 ml
- Trigger solution: TS12-1, 12 ml
- 15 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 CRP 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”.
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 “Dog CRP”.
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”.

[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.

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.

1. From the protocol manager select “Measure” and the program you would like to modify.
2. Select “New” and “Copy Protocol”.
3. Increase the Replicates as desired.
4. Create a new protocol name.
5. Select “Protocols” and save the new protocol.

SAMPLE PREP

This assay was designed for measurement of CRP in serum or plasma. In studies at Veterinary Biomarkers, Inc., we found CRP levels in serum ranging from 0 to >500 μg/ml. Testing at a dilution of 10,000-fold allows measurement of levels ranging from 0 to 100 μg/ml. A 10,000-fold dilution can be obtained as follows.

1. For each sample to be tested dispense 0.495 ml of diluent CSD50-1 into two tubes.
2. Mix 5.0 μl of serum (or plasma) with 0.495 ml of diluent in the first tube. This gives a 100-fold dilution.
3. Mix 5.0 μl of the 100-fold diluted sample with 0.495 ml of diluent in the second tube. This gives a 10,000-fold dilution.

STANDARD PREP

1. The CRP standard is provided in lyophilized form.
2. Add the volume of CSD50-1 diluent indicated on the vial label and mix. Use a vortex mixer if available. This provides the 10 ng/ml standard.
3. Use the standard within 30 min of reconstitution.

CONJUGATE 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 10 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 or blank.
3. Pipet 100 μl of the 10 ng/ml CRP 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. Avoid creating bubbles.
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. CRP concentrations are automatically calculated.
11. After measurement of the last sample, select “End”.
12. Results will be saved but may be exported as Excel or pdf files via a USB stick.

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

[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.

ASSAY PERFORMANCE

Typical data: The table below shows results from an assay in which the zero standard (blank), 10 ng/ml standard, and three serum samples were tested as singlets.

Veterinary Biomarkers, Inc.

Dog CRP Exp#002 12.12.2019 18:17:54

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			3,670	0
Standard			169,866	0.01
1	10000		164,094	96.527
3	1000		44,719	2.4699
5	10000		47,629	26.45

Reproducibility. Five serum samples with different CRP levels were tested in triplicate (Table 1) or as singlets (Table 2) in three separate assays at a 10,000-folod dilution. Levels of CRP are reported as µg/ml (mean ± SD, in Table 1). We find that samples need only be tested as singlets in a single assay.

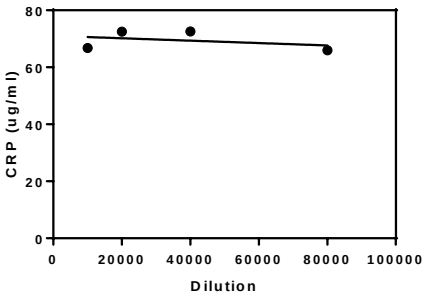
Table 1.

Sample	Assay 1	Assay 2	Assay 3
1	87.7±3.1	88.5±2.7	86.7±1.6
2	11.1±0.9	10.7±1.3	10.9±0.8
3	3.7±0.2	3.2±0.8	3.6±0.1
4	17.9±0.6	17.1±0.8	17.5±0.7
5	25.9±1.0	26.1±1.2	25.6±1.0

Table 2.

Sample	Assay 1	Assay 2	Assay 3
1	88.2	87.0	85.1
2	10.2	10.8	11.8
3	3.7	3.5	3.5
4	17.3	16.9	18.2
5	26.4	25.8	24.5

Linearity: To assess the linearity of the assay, a serum sample containing CRP at a concentration of 69.5 µg/ml was serially diluted with diluent CSD50-1 to produce values within the dynamic range of the assay.



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