COW CRP SPARCL™ ASSAY
Life Diagnostics, Inc., Catalog Number: CRP-SP-11

INTRODUCTION

CRP (C-reactive protein) is an acute phase protein that is elevated in cow serum because of injury, infection, and disease. Levels increase in milk due to mastitis (refs 1&2). In studies at Life Diagnostics, we found CRP levels of 14.6±3.4 μg/ml (mean±SD, n=3) in normal serum. In serum from cows with mastitis, levels were 104.7±122.3 μg/ml (mean±SD, n=3). In milk, we found CRP levels of ~30 μg/ml in normal samples. Levels were 97±48 μg/ml (mean±SD, n=4) in milk with somatic cell counts of 200,000-300,000.

PRINCIPLE OF THE ASSAY

The cow CRP SPARCL™[1] (Spatial Proximity Analyte Reagent Capture Luminescence, ref 3) assay uses two different affinity purified CRP-specific antibodies. One is conjugated to horseradish peroxidase (HRP) and the other is conjugated to acridan, a chemiluminescent substrate. When the HRP and acridan conjugated antibodies bind to CRP 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 CRP concentrations.

The HRP and acridan conjugated antibodies provided with the kit are mixed with standards and diluted samples in wells of the 96-well SPARCL™ plate provided with the kit[2]. 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 CRP is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-CRP HRP conjugate **Store ≤ -70°C**
- Anti-CRP acridan conjugate **Store ≤ -70°C**
- Cow CRP stock (2 vials) **Store ≤ -70°C**
- Diluent: CSD50-1, 2 x 50 ml
- Trigger solution: TS7-1, 7 ml
- White SPARCL™ plate (12 x 8-well)
- Clear untreated 96-well plate

Materials required but not provided:

- Precision pipettes and tips
- Polypropylene microcentrifuge tubes
- Vortex mixer
- Plate incubator/shaker
- Luminometer capable of simultaneous injection/measurement
- Graphing software

STORAGE

Store the HRP conjugate, acridan conjugate and CRP stock at -70°C (they may be stored at -20°C for one week). The remainder of the kit should be stored at 4°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 above.

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 (25°C) before use.
3. It is important that standards and samples be added to the SPARCL™ plate quickly. If testing large numbers of samples, rather than pipetting standards and samples directly into the white SPARCL™ plate using a single channel pipettor, we recommend the following. First, pipette an excess volume of standards and samples into appropriate wells of the clear 96-well plate. Then use an 8- or 12-channel multipipettor to quickly and efficiently transfer 50 μl aliquots to the appropriate wells of the white SPARCL™ plate. The wells of the clear plate hold a maximum volume of 300 μl.
4. Follow the sequence of events below when running the assay.

Prime and program the Luminometer
⇩
Prepare standards and diluted samples
⇩
Prepare HRP + Acridan conjugate mix
⇩
Add HRP + Acridan conjugate mix to the wells (25 μl)
⇩
Add standards and samples to the wells (50 μl)
⇩
Incubate plate at 150 rpm/25°C for 30 min
⇩
Measure luminescence after injection of Trigger (37.5 μl)

STANDARD PREPARATION

The cow CRP stock is comprised of lyophilized cow acute phase serum. The CRP content was determined by reference to purified cow CRP prepared at Life Diagnostics, Inc.

1. Reconstitute the lyophilized cow CRP stock with water as described on the vial label. Mix gently until dissolved. The concentration of CRP in the reconstituted stock is indicated on the label.
2. Label 8 polypropylene tubes as 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, 0.98 and 0.49 ng/ml.
3. Into the tube labeled 62.5 ng/ml, pipette the volume of diluent detailed on the CRP stock vial label. Then add the indicated volume of CRP stock and mix gently. This provides the 62.5 ng/ml standard.
4. Dispense 150 μl of diluent into the tubes labeled 250, 125, 62.5, 31.25, 15.63, 7.81 and 3.91 ng/ml.
5. Pipette 150 μl of the 62.5 ng/ml CRP standard into the tube labeled 31.25 ng/ml and mix. This provides the 31.25 ng/ml CRP standard.
6. Similarly prepare the remaining standards by serial dilution.

Use the standards within one hour of preparation.

SAMPLE PREPARATION

Serum or heparinized plasma should be prepared as quickly as possible after blood collection. Do not use EDTA or citrated plasma unless samples are diluted at least 100-fold (EDTA and citrate inhibit HRP causing false low CRP values). All samples should be similarly processed (i.e., storage times and temperatures should be the same). If samples cannot be assayed immediately, they should be frozen at or below –20°C. Avoid repeated freeze-thaws.

Serum and plasma. Because the cow CRP SPARCL assay uses a homogeneous format, it is susceptible to a prozone or "hook effect" at high CRP concentrations. We found that if serum samples were tested at dilutions of 4000-fold or greater the prozone effect was eliminated.

Milk. In order to avoid a matrix effect, we found it necessary to dilute milk at least 16-fold with diluent. When analyzing milk with low CRP levels we used a modified standard curve with a range of 15.63 to 0.12 ng/ml).

Because CRP levels depend on the degree of acute phase response and the timing of sample collection, optimal dilutions must be determined by the end user.

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.

[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.

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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 4°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.

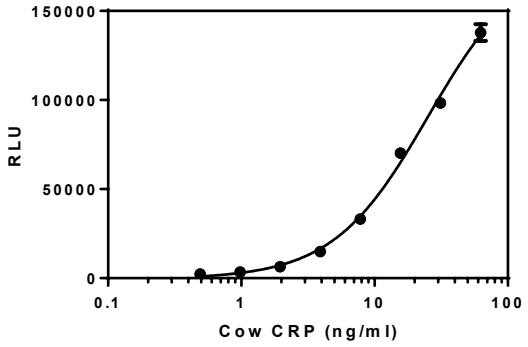
CALCULATION OF RESULTS

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. We routinely use the sum of RLU values from a 100-980 ms data collection window.
2. Determine the sum of RLU values within the data collection window for the standards and samples.
3. Using graphing software, construct a standard curve by plotting the sum of the RLU values for the standards versus the log10 of the CRP concentration and fit to a sigmoidal, 4PL model.
4. Derive the corresponding concentration of CRP in the samples from the standard curve (remember to derive the concentration from the antilog).
5. Multiply the derived concentration by the dilution factor to determine the concentration of CRP in the original sample.
6. If the sum of the RLU values of diluted samples fall outside the standard curve, samples should be appropriately diluted and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with sum of RLU versus $\log_{10}$ CRP concentration is shown below. This curve is for illustration only and should not be used to calculate unknowns. A standard curve should be run with each experiment.

CRP (ng/ml)	RLU
62.5	137871
31.25	98288
15.63	70190
7.81	33189
3.91	14983
1.95	6414
0.98	3491
0.49	2252



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2. Thomas FC. et al. Mastitomics, the integrated omics of bovine milk in an experimental model of Streptococcus uberis mastitis: 1. High abundance proteins, acute phase proteins and peptidomics. Mol Biosyst. 12(9):2735-2747 (2016)
3. Akhavan-Tafti H. et al. A homogeneous chemiluminescent immunoassay method. J Am Chem Soc. 20;135(11):4191-4 (2013)

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For technical assistance please email us at techsupport@lifediagnostics.com