DOG LACTOFERRIN SPARCL™ ASSAY
Life Diagnostics, Inc., Catalog Number: LF-SP-4

BACKGROUND

Lactoferrin (LF) is a non-heme iron binding glycoprotein found in milk, other secretory fluids and blood. As a component of host defense, it has antimicrobial and anti-inflammatory activity. In humans, it is used as a biomarker of intestinal inflammation (refs 1&2). The utility of LF as a biomarker of intestinal inflammation in dogs is currently under investigation.

PRINCIPLE OF THE ASSAY

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

The HRP and acridan conjugated antibodies provided with the kit are mixed with standards and diluted samples in wells of the 96-well white 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 LF is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-LF HRP stock. **Store ≤ -70°C**
- Anti-LF acridan stock. **Store ≤ -70°C**
- LF stock (1 vial). **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 tubes
- Vortex mixer
- Micro-Plate incubator/shaker
- Luminometer capable of simultaneous injection & measurement
- PC graphing software

STORAGE

Store the HRP conjugate, acridan conjugate and LF stock at or below -70°C. 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

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 LF stock is comprised of pure LF diluted in a carrier protein matrix.

1. Thaw the LF stock shortly before use.
2. Label 7 polypropylene tubes as 5, 2.5, 1.25, 0.625, 0.313, 0.156 and 0.078 ng/ml.
3. Into the tube labeled 5 ng/ml, pipette the volume of diluent detailed on the stock vial label. Then add the indicated volume of stock and mix gently. This provides the 5 ng/ml standard.
4. Dispense 150 µl of diluent into the tubes labeled 2.5, 1.25, 0.625, 0.313, 0.156 and 0.078 ng/ml.
5. Pipette 150 µl of the 5 ng/ml LF standard into the tube labeled 2.5 ng/ml and mix. This provides the 2.5 ng/ml LF standard.
6. Similarly prepare the remaining standards by two-fold serial dilution.

Please Note: If future use of the LF stock is intended, it should be stored frozen at or below -70°C.

[1] The SPARCL™ technology was developed by Lumigen Corp.

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

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SAMPLE PREPARATION

We found LF levels of 13.2±23.7 ng/ml (mean±SD, n=37) in serum from sick dogs. Levels ranged from undetectable to >100 ng/ml. Optimal dilutions should be determined by the end user but we suggest an initial dilution of 20-fold (10 µl of serum plus 190 µl of diluent). To avoid matrix effects, do not use dilutions lower than 20-fold. Plasma should not be used. Only use the dilution buffer provided with the kit.

CONJUGATE MIX PREPARATION

Instructions for preparation of the conjugate mix are detailed on the box that contains the HRP and acridan conjugates. Prepare the mix shortly before use using the diluent provided with the kit (CSD50-1).

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 at least 1 ml of trigger solution.
3. Place the injection needle into the injection port, (necessary for BMG luminometers).
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 2-8°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.

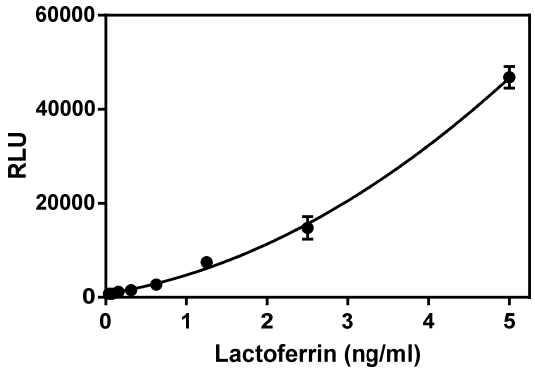
CALCULATION OF RESULTS

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 RLU values (RLU) for the standards versus the LF concentration in ng/ml.
4. Fit the data using graphing software. We find that a second order polynomial model works well.
5. Derive the corresponding concentration of LF in the samples from the standard curve.
6. Multiply the derived concentration by the dilution factor to determine the actual concentration of LF in the serum sample.
7. 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 LF concentrations on the X-axis is shown below. This curve is for illustration only and should not be used to calculate unknowns. A standard curve must be run with each experiment.

LF (ng/ml)	RLU
5.0	46807
2.5	14787
1.25	7492
0.625	2702
0.313	1542
0.156	1198
0.078	785



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