

COW LACTOFERRIN SPARCL™ ASSAY

Life Diagnostics, Inc., Catalog Number: LF-SP-11

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. It is an excellent biomarker of mastitis in cattle (refs 1&2). Lactoferrin levels may range from less than 0.05 mg/ml in normal milk to more than 8 mg/ml in milk from animals with mastitis (refs 1&2).

PRINCIPLE OF THE ASSAY

The cow LF SPARCL™¹ (Spatial Proximity Analyte Reagent Capture Luminescence, ref 3) assay uses two 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 kit². 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-Lactoferrin HRP conjugate **Store ≤ -70°C**
- Anti-Lactoferrin acridan conjugate **Store ≤ -70°C**
- Lactoferrin stock **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
- Glacial acetic acid (if testing milk)
- Luminometer capable of simultaneous injection/measurement
- Curve fitting software

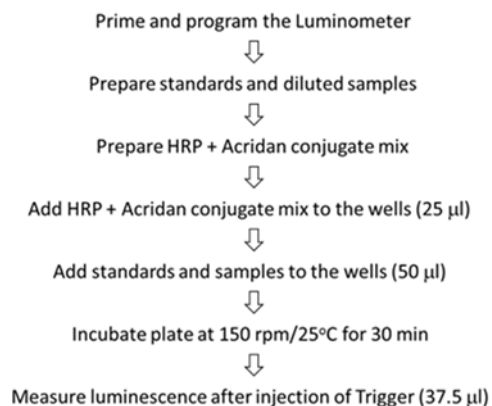
STORAGE

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.



STANDARD PREPARATION

The LF stock is comprised of pure cow LF³ in a carrier protein matrix.

1. Thaw the LF stock shortly before use.
2. Label 8 polypropylene tubes as 100, 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 ng/ml.
3. Into the tube labeled 100 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 100 ng/ml standard.
4. Dispense 150 µl of diluent into the tubes labeled 50, 25, 12.5, 6.25, 3.13, 1.56 and 0.78 ng/ml.
5. Pipette 150 µl of the 100 ng/ml LF standard into the tube labeled 50 ng/ml and mix. This provides the 50 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.

³ The cow LF used in the standard was purified at Life Diagnostics, Inc.

SAMPLE PREPARATION

Serum. Using the cow LF SPARCL™ assay, we found LF levels of 180 ± 71 $\mu\text{g/ml}$ (mean \pm SD, n=9) in serum from healthy cows. Levels were 839 ± 820 $\mu\text{g/ml}$ (mean \pm SD, n=9) in serum from cows that were sick for reasons other than mastitis. We recommend diluting serum 60-fold prior to assay (2.5 μl of serum plus 147.5 μl of diluent CSD50-1). Matrix effects may occur at dilutions lower than 60-fold.

Milk. Raw milk can be used in this assay but dilutional linearity is poor. We therefore recommend that whey prepared from raw milk be used instead. Whey can be rapidly prepared as follows.

1. Pipette 0.50 ml of raw milk into a 1.5 ml microcentrifuge tube.
2. Add 3.50 μl of glacial acetic acid and mix by vortexing.
3. Incubate for 15 minutes at room temperature.
4. Centrifuge for 5 minutes at 14,000 rpm in a microcentrifuge.
5. Carefully remove the supernatant (whey) using a 100 or 200 μl pipettor. Be careful to not aspirate the fat layer, if present.
6. Immediately dilute the whey sample at least 500-fold with diluent CSD50-1 (2.0 μl of whey plus 998 μl of CSD50-1)
7. As described below, further dilution may be necessary prior to testing.

In studies at Life Diagnostics, we found LF levels of 24.6 ± 14 $\mu\text{g/ml}$ (mean \pm SD, n=2), 116 ± 54 $\mu\text{g/ml}$ (mean \pm SD, n=4), and 243 ± 107 $\mu\text{g/ml}$ (mean \pm SD, n=4) in milk with somatic cell counts of 4,000, 100,000 and 200-300,000 respectively. All whey samples that we tested gave values within range of the standard curve at a dilution of 8,000-fold (25 μl of the 500-fold dilution plus 175 μl of CSD50-1). It may be necessary for optimal sample dilutions to be determined by the end user. Do not use buffers other than CSD50-1 for dilution.

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.

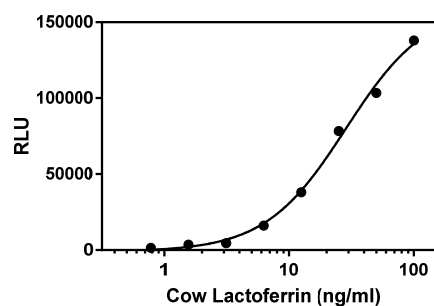
CALCULATION OF RESULTS

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 the RLU values for the standards versus the \log_{10} of the LF concentration and fit to a sigmoidal, 4PL model.
4. Derive the corresponding concentration of LF 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 LF 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} LF 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.

LF (ng/ml)	RLU
100	147294
50	115475
25	84176
12.5	42860
6.25	18657
3.13	7844
1.56	3491
0.78	1947



REFERENCES

1. Kawai K, Hagiwara S, Anri A, Nagahata H. Lactoferrin concentration in milk of bovine clinical mastitis. *Vet Res Commun.* 23(7):391-398 (1999)
2. Harmon RJ et al. Changes in lactoferrin, immunoglobulin G, bovine serum albumin, and a-lactalbumin during acute experimental and natural coliform mastitis in cows. *Infection and Immunity.* 13:533-542 (1976)
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
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