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

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated in cow serum and milk as a result of injury, infection and disease. In studies at Life Diagnostics, we found that serum haptoglobin levels range from ~0.1 μg/ml in healthy cows to greater than 4 mg/ml in sick cows. Levels were ~50 ng/ml in milk from healthy cows and greater than 2000 ng/ml in milk from cows with mastitis (SCC = 200-300 cells/ml). Haptoglobin therefore provides a useful biomarker of inflammation and disease in cows.

PRINCIPLE OF THE ASSAY

The cow haptoglobin SPARCL™[1] (Spatial Proximity Analyte Reagent Capture Luminescence, ref 1) assay uses two cow haptoglobin-specific antibodies. One is conjugated to horseradish peroxidase (HRP), the other is conjugated to acridan, a chemiluminescent substrate. When the HRP and acridan conjugated antibodies bind to haptoglobin 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 haptoglobin 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 haptoglobin is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-cow haptoglobin HRP conjugate stock. **Store ≤ -70°C**
- Anti-cow haptoglobin acridan conjugate stock. **Store ≤ -70°C**
- Cow haptoglobin stock (3 vials). **Store ≤ -70°C**
- Diluent (CSD50-1), 2 x 50 ml
- Trigger solution, 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 haptoglobin stock at -70°C (they may be stored at -20°C for one week). 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 above.

GENERAL INSTRUCTIONS

The dilution buffer and 8-well strips used in the assay should be allowed to reach room temperature (25°C) before use.

STANDARD PREPARATION

The cow haptoglobin stock is comprised of purified cow haptoglobin lyophilized in a carrier protein matrix.

1. Reconstitute the lyophilized stock with diluent as described on the vial label. Mix gently until dissolved.
2. Label 6 polypropylene tubes[3] as 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 ng/ml.
3. Into the tube labeled 62.5 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 62.5 ng/ml standard.
4. Dispense 150 μl of diluent into the tubes labeled 31.25, 15.63, 7.81, 3.91 and 1.95 ng/ml.
5. Pipette 150 μl of the 62.5 ng/ml haptoglobin standard into the tube labeled 31.25 ng/ml and mix. This provides the 31.25 ng/ml haptoglobin standard.
6. Similarly prepare the remaining standards by two-fold serial dilution.

Please Note: Use the standards within one hour of preparation.

SAMPLE PREPARATION

Serum & Plasma. Serum or heparinized plasma should be prepared as quickly as possible after blood collection. Do not use EDTA or citrate plasma unless samples are diluted at least 100-fold (EDTA and citrate inhibit HRP causing false low haptoglobin 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.

The cow haptoglobin SPARCL assay uses a homogeneous format and is therefore susceptible to a prozone or "hook effect" at high haptoglobin concentrations. Because serum levels can range from 0.1 to >4 mg/ml, unknown samples should be tested at several different dilutions in order to ensure that values fall within range of the standard curve and to eliminate false low values caused by a prozone effect. We found that if samples were tested at dilutions of 20, 2,000 and 80,000-fold most serum samples fell within range of the standard curve. However, because haptoglobin levels depend on

[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.

[3] Dilutions of standards can be performed in wells A1-A8 of the clear untreated 96-well plate provided with the kit. This allows rapid transfer of standards to the white SPARCL™ plate using a multipipettor. Diluted samples can also be first aliquoted into appropriate wells of the clear polystyrene plate and subsequently transferred to the SPARCL™ plate with a multipipettor. If using this method, ensure that an excess volume is aliquoted into the clear plate in order to ensure transfer of 50 μl aliquots to the SPARCL™ plate.

the magnitude of acute phase response and the timing of sample collection, optimal dilutions must be determined by the end user. Dilutions of 20, 2,000 and 80,000-fold can be achieved using the following procedure for each sample to be tested:

1. Dispense 190 µl, 198 µl and 195 µl of diluent into separate tubes.
2. Pipette and mix 10.0 µl of the serum sample into the tube containing 190 µl of diluent. This provides a 20-fold dilution.
3. Mix 2.0 µl of the 20 fold diluted sample with the 198 µl of diluent in the second tube. This provides a 2,000-fold dilution.
4. Mix 5 µl of the 2,000-fold diluted sample with 195 µl of diluent in the third tube to give an 80,000-fold dilution.

Milk. Unpasteurized milk can be tested at dilutions of 10-fold or greater. Values within range of the standard curve can usually be obtained if samples are tested at dilutions of 10- and 100-fold.

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.
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 50.0 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.

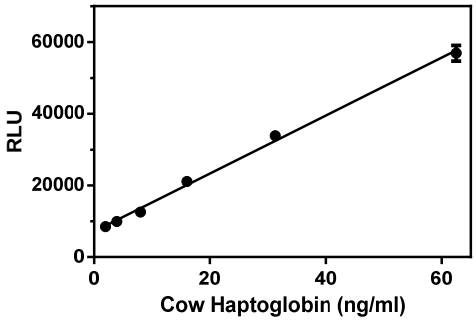
CALCULATION OF RESULTS

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.
2. Using graphing software, construct a standard curve by plotting the luminescence (RLU) for the standards versus haptoglobin concentration in ng/ml. We fit data to either a two site binding equation or a second order polynomial equation.
3. Derive the corresponding concentration of haptoglobin in the samples from the standard curve.
4. Multiply the derived concentration by the dilution factor to determine the actual concentration of haptoglobin in the serum or plasma sample.
5. 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 haptoglobin concentrations on the X-axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. A standard curve must be run for each experiment.

Haptoglobin (ng/ml)	RLU
62.5	56884
31.25	33885
15.63	21060
7.81	12597
3.91	9971
1.95	8472



REFERENCES

1. Akhavan-Tafti H. et al. A homogeneous chemiluminescent immunoassay method. J Am Chem Soc. 20;135(11):4191-4 (2013)

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