

HORSE HAPTOGLOBIN SPARCL™ ASSAY

Life Diagnostics, Inc., Catalog Number: HAPT-SP-14

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated in the serum of most mammals during injury, infection and disease. In horses, serum haptoglobin increases approximately 10-fold during the acute phase response (refs 1-2), making it a useful inflammatory biomarker.

PRINCIPLE OF THE ASSAY

The horse haptoglobin SPARCL™¹ (Spatial Proximity Analyte Reagent Capture Luminescence, ref 3) assay uses two horse 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 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 haptoglobin is proportional to luminescence and is derived from a standard curve.

MATERIALS AND COMPONENTS

Materials provided with the kit:

- Anti-haptoglobin HRP conjugate **Store ≤ -70°C**
- Anti-haptoglobin acridan conjugate **Store ≤ -70°C**
- Haptoglobin stock (3 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:

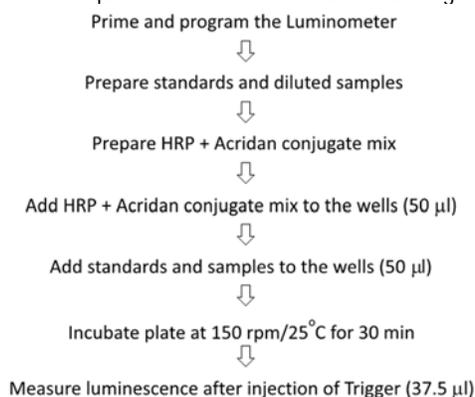
- Precision pipettes and tips
- Polypropylene microcentrifuge tubes
- Vortex mixer
- Plate incubator/shaker
- Luminometer capable of simultaneous injection/measurement
- Curve fitting software

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.

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 horse haptoglobin stock is comprised of horse serum lyophilized in a carrier protein matrix. The haptoglobin content was determined by reference to purified horse haptoglobin prepared at Life Diagnostics, Inc.

1. Reconstitute the lyophilized stock with diluent as described on the vial label. Mix gently until dissolved.
2. Label 8 polypropylene tubes as 180, 90, 45, 22.5, 11.25, 5.63, 2.81 and 1.41 ng/ml.
3. Into the tube labeled 180 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 180 ng/ml standard.
4. Dispense 150 µl of diluent into the tubes labeled 90, 45, 22.5, 11.25, 5.63, 2.81 and 1.41 ng/ml.
5. Pipette 150 µl of the 180 ng/ml haptoglobin standard into the tube labeled 90 ng/ml and mix. This provides the 90 ng/ml haptoglobin standard.
6. Similarly prepare the remaining standards by two-fold serial dilution.

Use the standards within one hour of preparation.

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.

SAMPLE PREPARATION

Serum or plasma should be prepared as quickly as possible after blood collection. If samples cannot be assayed immediately they should be frozen at or below -20°C . Avoid repeated freeze-thaws.

The horse haptoglobin SPARCL assay uses a homogeneous format and is therefore susceptible to a prozone or "hook effect" at high haptoglobin concentrations. In normal horse serum, we found haptoglobin levels of $301 \pm 130 \mu\text{g/ml}$. We found that if samples were tested at a dilution of 8000-fold, all samples fell within range of the standard curve and a prozone effect could be avoided. However, because haptoglobin levels depend on the magnitude of acute phase response and the timing of serum collection, optimal dilutions should be determined by the end user.

A dilution of 8000-fold can be achieved as follows:

1. Dispense 98 μl and 318 μl of diluent into separate tubes.
2. Pipette and mix 2.0 μl of the sample into the tube containing 98 μl of diluent. This provides a 50-fold dilution.
3. Mix 2.0 μl of the 50-fold diluted sample with the 318 μl of diluent in the second tube. This provides an 8000-fold dilution.

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 $2-8^{\circ}\text{C}$.
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.

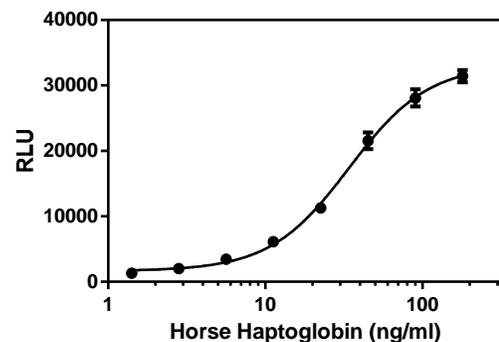
CALCULATION OF RESULTS

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. 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 haptoglobin concentration and fit to a sigmoidal, 4PL model.
4. Derive the corresponding concentration of haptoglobin 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 haptoglobin 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} haptoglobin 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.

Haptoglobin (ng/ml)	RLU
180	31428
90	28090
45	21556
22.5	11274
11.25	6119
5.63	3465
2.81	2024
1.41	1315



REFERENCES

1. Tiara T, et al. Equine haptoglobin: isolation, characterization, and the effects of ageing, delivery and inflammation on its serum concentration. *J Vet Med Sci.* 54:435-42 (1992)

2. Kent JE and Goodall J. Assessment of an immunoturbidimetric method for measuring equine serum haptoglobin concentrations. *Equine Vet J.* 23:59-66 (1991)
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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