

PIG HAPTOGLOBIN SPARCL™ ASSAY

Life Diagnostics, Inc., Catalog Number: HAPT-SP-9

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated in pig serum during injury, infection and disease. Levels increase approximately five-fold during the acute phase response, making it a useful inflammatory biomarker (refs 1-3).

PRINCIPLE OF THE ASSAY

The pig haptoglobin SPARCL™¹ (Spatial Proximity Analyte Reagent Capture Luminescence, ref 4) assay uses two pig 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-pig haptoglobin HRP conjugate stock. **Store ≤ -70°C**
- Anti-pig haptoglobin acridan conjugate stock. **Store ≤ -70°C**
- Pig 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 pig haptoglobin stock is comprised of pig serum lyophilized in a carrier protein matrix. The haptoglobin content was determined by reference to purified pig haptoglobin prepared at Life Diagnostics, Inc.

1. Reconstitute the stock with diluent as described on the vial label. Mix gently until dissolved.
2. Label 8 polypropylene tubes³ as 200, 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 ng/ml.
3. Into the tube labeled 200 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 200 ng/ml standard.
4. Dispense 150 µl of diluent into the tubes labeled 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 ng/ml.
5. Pipette 150 µl of the 200 ng/ml haptoglobin standard into the tube labeled 100 ng/ml and mix. This provides the 100 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 pig 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 >7 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 a dilution of 80,000-fold, most serum samples fell within range of the standard curve. However, because haptoglobin levels depend on the degree of acute phase response and the timing of serum collection, optimal dilutions must be determined by the end user.

A dilution of 80,000-fold can be achieved as follows:

1. Dispense 98 µl, 198 µl and 150 µl of diluent into separate tubes.

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 complete transfer of 50 µl aliquots to the SPARCL™ plate.

2. Pipette and mix 2.0 μl of the serum 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 198 μl of diluent in the second tube. This provides a 5,000-fold dilution.
4. Mix 10 μl of the 5,000-fold diluted sample with 150 μl of diluent in the third tube to give an 80,000-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°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

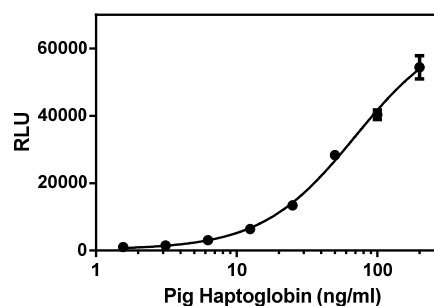
1. Before calculating results, review the raw data. If artefacts (RLU spikes) are apparent immediately after injection of trigger solution, eliminate that portion of the luminescence profile from analysis for all wells.
2. Using graphing software, construct a standard curve by plotting the luminescence (RLU) for the standards versus Log_{10} of the haptoglobin concentration.

3. Fit data using a sigmoidal four parameter logistic equation.
4. Derive the corresponding concentration of haptoglobin in the samples from the standard curve (convert the antilog to concentration in ng/ml).
5. Multiply the derived concentration by the dilution factor to determine the actual concentration of haptoglobin in the serum or plasma sample.
6. 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. A standard curve must be run with each experiment.

Haptoglobin (ng/ml)	RLU
200	54384
100	40312
50	28348
25	13415
12.5	6382
6.25	3059
3.13	1459
1.56	1011



REFERENCES

1. Kreuzer S et. al. No beneficial effects evident for *Enterococcus faecium* NCIMB 10415 in weaned pigs infected with *Salmonella enterica* Typhimurium DT104. *Appl Environ Microbiol.* 78(14):4816-4825
2. Pomorska-Mol M, Markowska-Daniel I and Kwit K. Acute phase protein response in pigs experimentally co-infected with swine influenza virus and *Bordetella bronchiseptica*. *Centr Eur J Immunol* 37(3):221-226 (2012)
3. Pomorska-Mol M, Kwit K and Markowska-Daniel I. Major acute phase proteins in pig serum from birth to slaughter. *Bull Vet Inst Pulawy* 56:553-557 (2012)
4. Akhavan-Tafti H. et al. A homogeneous chemiluminescent immunoassay method. *J Am Chem Soc.* 20;135(11):4191-4 (2013)

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