INTRODUCTION

Haptoglobin-like protein (referred to here as haptoglobin) was identified in proteomic studies as a protein that is increased in Atlantic salmon with cardiomyopathy syndrome (ref 1) and inflammation (ref 2). It was also found to be upregulated in rainbow trout after challenge with Flavobacterium psychrophilum (ref 3). In studies at Life Diagnostics, we found that haptoglobin levels were undetectable in healthy rainbow trout. Levels increased to ~8 μg/ml six days after Yersinia ruckeri infection.

PRINCIPLE OF THE ASSAY

The assay uses a polyclonal antibody that recognizes Atlantic salmon and rainbow trout haptoglobin. Unconjugated antibody is coated on wells of a microtiter plate and used for capture. Horseradish peroxidase (HRP) conjugated antibody is used for detection. Standards and diluted samples (100 μl) are incubated in the antibody coated microtiter wells for one hour. After washing the wells, HRP-conjugate (100 μl) is added and incubated for 45 minutes. If haptoglobin molecules are present, they are sandwiched between the capture and detection antibodies. The wells are then washed to remove unbound HRP-conjugate. TMB is added and incubated for 20 minutes. If haptoglobin is present, a blue color develops. Color development is stopped by addition of Stop Solution, changing the color to yellow. Absorbance is measured at 450 nm. The concentration of haptoglobin is proportional to absorbance and is derived from a standard curve.

MATERIALS

Materials provided with the kit:

- Anti-haptoglobin coated plate (12 x 8-well strips)
- 2x HRP conjugate, 7 ml
- Haptoglobin stock, 2 vials. **Store at -20°C**
- 20x Wash Solution: TBS50-20, 50 ml
- Diluent: YD50-1, 2 x 50 ml
- TMB: TMB11-1, 11 ml
- Stop Solution: SS11-1, 11 ml

Materials required but not provided:

- Pipettors and tips
- Distilled or deionized water
- Polypropylene tubes or 96-well polystyrene plates
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm
- Graphing software

STORAGE

Store the standard vial at -20°C. The remainder of the kit should be stored at 4°C and the microtiter plate should be kept in a sealed bag with desiccant. The kit will remain stable for six months from the date of purchase.

GENERAL INSTRUCTIONS

1. All reagents should be allowed to reach room temperature before use.
2. Reliable and reproducible results will be obtained when the assay is conducted with a complete understanding of the instructions and with adherence to good laboratory practice.
3. It is important that standards and samples be added to the ELISA plate quickly. If testing large numbers of samples, rather than pipetting standards and samples from individual tubes into the ELISA plate, we recommend the following: pipette an excess volume of standards and samples into wells of a blank polystyrene 96-well plate[1]. Then use an 8 or 12-channel multi-pipettor to quickly transfer 100 μl aliquots to the wells of the antibody-coated plate.
4. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
5. Laboratory temperature will influence absorbance readings. The assay was calibrated using a shaking incubator set at 150 rpm and 25°C. Performing the assay at lower temperatures and mixing speeds may result in lower absorbance values.

WASH SOLUTION

The wash solution is provided as a 20x stock. Prior to use, dilute the contents of the bottle (50 ml) with 950 ml of distilled or deionized water. Unused wash buffer may be stored at 4°C for one week.

DILUENT

The diluent is formulated for measurement of haptoglobin in trout and salmon serum or plasma. It is supplied ready to use. DO NOT substitute other buffers.

STANDARD

1. The stock is lyophilized. Reconstitute it with the volume of diluent shown on the vial label and prepare the 40 ng/ml standard as described on the vial label.
2. Label seven polypropylene tubes as 20, 10, 5, 2.5, 1.25, 0.625, and 0 ng/ml. Dispense 0.25 ml of diluent into each.
3. Pipette 0.25 ml of the 40 ng/ml haptoglobin standard into the tube labeled 20 ng/ml and mix. This provides the 20 ng/ml haptoglobin standard.
4. Similarly prepare the 10 – 0.625 ng/ml standards by two-fold serial dilution.

Discard the stock after use.

[1] Standards and sample dilutions may also be prepared directly in a blank polystyrene plate.

HRP CONJUGATE

For each 8-well strip used in the assay, mix 0.5 ml of 2x HRP conjugate with 0.5 ml of YD50-1 diluent. Use 100 µl per well.

SAMPLES

In studies at Life Diagnostics, we found haptoglobin levels ranging from 0 to ~8 µg/ml in rainbow trout plasma. Optimal dilutions should be determined empirically. However, we suggest that samples initially be tested at a dilution of 100-fold (2.5 µl of serum or plasma mixed with 247.5 µl of diluent). Ideally dilutions should be performed in polystyrene 96-well plates (not provided). This allows quick and easy transfer of diluted samples to the antibody-coated plate using 8- or 12-channel multi-pipettors.

PROCEDURE

1. Secure the desired number of 8-well strips in the cassette. Unused strips should be stored in a sealed bag with desiccant at 4°C.
2. Dispense 100 µl of standards and samples into the wells.
3. Incubate on a plate shaker at 150 rpm and 25°C for 1 hour.
4. Empty and wash the microtiter wells 5x with 1x wash solution using a plate washer (400 µl/well).
5. Dispense 100 µl of 1x HRP conjugate into the wells.
6. Incubate on a plate shaker at 150 rpm and 25°C for 45-minutes.
7. Empty and wash the microtiter wells 5x with 1x wash solution using a plate washer (400 µl/well).
8. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
9. Dispense 100 µl of TMB into each well.
10. Incubate on an orbital micro-plate shaker at 150 rpm at 25°C for 20 minutes.
11. After 20-minutes, stop the reaction by adding 100 µl of Stop solution to each well.
12. Gently mix. It is important to make sure that all the blue color changes to yellow.
13. Read absorbance at 450 nm[2] with a plate reader within 5 minutes.

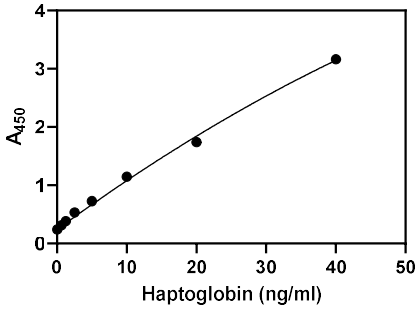
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the haptoglobin concentration.
2. Fit the standard curve using graphing software. We suggest using a second order polynomial (quadratic)equation.
3. Derive the concentration of haptoglobin in the samples.
4. Multiply the derived concentration by the dilution factor to determine the concentration in the sample.
5. If the absorbance values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

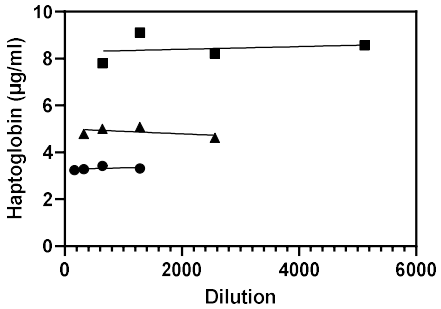
A typical standard curve is shown below. This curve is for illustration only.

Haptoglobin (ng/ml)	A_{450}
40	3.166
20	1.743
10	1.150
5	0.727
2.5	0.535
1.25	0.385
0.625	0.313
0	0.241



PERFORMANCE

Linearity: To assess the linearity of the assay, three rainbow trout plasma samples with haptoglobin concentrations of 3.3, 4.9, and 8.4 µg/ml were serially diluted to produce values within the dynamic range of the assay.



[2] If absorbance of the high standard is ≥4 when measured at 450 nm, absorbance of all standards and samples should be read at 405 nm.

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