INTRODUCTION

Haptoglobin is an acute phase protein that is elevated in cow serum due to injury, infection, or disease. Normal serum levels of cow Haptoglobin range from 0 – 50 μg/ml, increasing up to 4 mg/ml during the acute phase response. Haptoglobin is also present in milk. Studies at Life Diagnostics indicate that levels increase from 11.7±0.30.9 μg/ml (n=20, range 0 – 119.8 μg /ml) in normal milk to 335±495 μg/ml (n=20, range 0 – 1.39 mg/ml) in milk from cows with mastitis.

PRINCIPLE OF THE ASSAY

The assay uses a monoclonal antibody (HAPT-11-3F7), that recognizes the haptoglobin heavy chain. Unconjugated antibody is used for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated antibody is used for detection. The same antibody can be used for capture and detection because each Haptoglobin heterodimer contains two heavy chains. Standards and diluted samples are incubated in the microtiter wells for 45 minutes. The wells are subsequently washed. HRP conjugate is added and incubated for 45 minutes. This results in Haptoglobin molecules being sandwiched between the immobilization 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 the 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:

- Haptoglobin antibody coated 96-well plate (12 x 8-well strips)
- HRP Conjugate, 11 ml
- Haptoglobin stock (lyophilized)
- 20x Wash solution: TBS50-20, 50 ml
- 10x Diluent: YD25-10, 25 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

The unused kit should be stored at 4°C and the microtiter plate should be kept in a sealed bag with desiccant. Kits 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 provided as a 10x stock. Dilute one volume of the 10x stock with nine volumes of distilled or deionized water to give the desired volume of working diluent.

STANDARDS

1. The Haptoglobin stock is provided lyophilized. Add the volume of distilled or deionized water indicated on the vial label and mix gently until dissolved to obtain a 2.5 μg/ml cow Haptoglobin stock. The reconstituted stock should be aliquoted and frozen at -20°C within 2 hours if additional use is intended.
2. Label 8 polypropylene or glass tubes as 250, 125, 62.5, 31.25, 15.6, 7.81, 3.91 and 0 ng/ml.
3. Dispense 450 μl of 1x diluent into the tube labeled 250 ng/ml and 250 μl of 1x diluent into the remaining tubes.
4. Pipette 50 μl of the 2.5 μg/ml Haptoglobin standard into the tube labeled 250 ng/ml and mix. This provides the 250 ng/ml standard.

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

5. Prepare the 125 ng/ml standard by diluting and mixing 250 µl of the 250 ng/ml standard with 250 µl of 1x diluent in the tube labeled 125 ng/ml.
6. Similarly prepare the 62.5, 31.25, 15.6, 7.81, and 3.91 standards by two-fold serial dilution.

HRP CONJUGATE

The HRP conjugate is supplied ready to use.

SAMPLES

Serum and Plasma: Haptoglobin is present in healthy cow serum at concentrations ranging from 0 - 50 µg/ml and can increase to over 4 mg/ml. To obtain values within range of the standard curve we suggest that serum samples be diluted 10,000-fold using the following procedure.

1. Dispense 198 µl and 247.5 µl of 1x diluent into separate tubes.
2. Pipette and mix 2.0 µl of the serum/plasma sample into the tube containing 198 µl of diluent. This provides a 100-fold dilution.
3. Mix 2.5 µl of the 100-fold diluted sample with the 247.5 µl of diluent in the second tube. This provides a 10,000-fold dilution.
4. Repeat this procedure for each sample to be tested.

Milk: In milk, we found values ranging from 0 to 1.5 mg/ml. We suggest that milk samples be tested at a 1,000-fold dilution, obtained as follows:

1. Dispense 198 µl and 225 µl of 1x diluent into separate tubes.
2. Pipette and mix 2.0 µl of the serum/plasma sample into the tube containing 198 µl of diluent. This provides a 100-fold dilution.
3. Mix 25 µl of the 100-fold diluted sample with the 225 µl of diluent in the second tube. This provides a 1,000-fold dilution.
4. Repeat this procedure for each sample to be tested.

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 appropriate wells. We recommend that standards and samples be tested in duplicate.
3. Incubate on a plate shaker set at 150 rpm and 25°C for 45 minutes.
4. Empty and wash the microtiter wells 5 times with 1x Wash Solution using a plate washer (400 µl/well). If necessary, strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
5. Dispense 100 µl of HRP conjugate into each well.
6. Incubate on a plate shaker set at 150 rpm and 25°C for 45 minutes.
7. Empty and wash the microtiter wells as described in step 4.
8. Dispense 100 µl of TMB into each well.
9. Incubate on an orbital micro-plate shaker at 150 rpm and 25°C for 20 minutes.
10. After 20 minutes stop the reaction by adding 100 µl of Stop Solution to each well.
11. Gently mix. It is important to make sure that all the blue color changes to yellow.
12. Read absorbance at 450 nm[2] with a plate reader within 5 minutes.

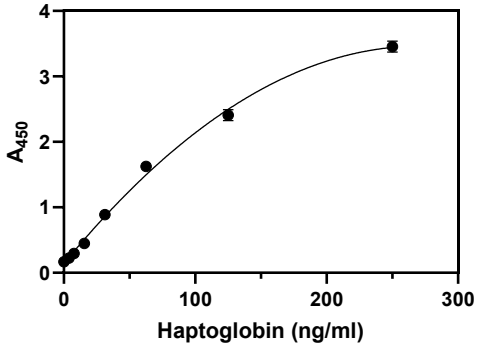
RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus the SAA concentration. We suggest using a second order polynomial (quadratic) equation.
2. Derive the concentration of SAA in the samples.
3. Multiply the derived concentration by the dilution factor to determine the concentration in the sample.
4. 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.

Hapt. (ng/ml)	A_{450}
250	3.538
125	2.286
62.5	1.201
31.25	0.658
15.63	0.397
7.81	0.244
3.91	0.150
0	0.055



[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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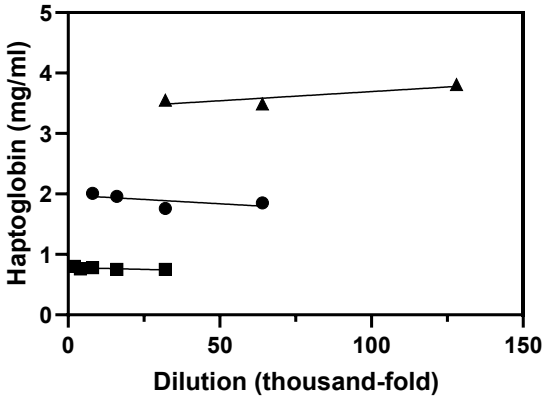
PERFORMANCE

Linearity: To assess the linearity of the assay, three serum samples with Haptoglobin concentrations of 0.77, 1.89, and 3.6 mg/ml were serially diluted to give values within range of the assay.



COMPARISON TO KIT HAPT-11

This ELISA kit (HAPT-11-N) replaces kit HAPT-11. The replacement was necessary because we found that new batches of the HRP-conjugate dilution buffer, gave unacceptably high background with the polyclonal antibody used in kit HAPT-11. Kit HAPT-11-N uses a monoclonal antibody (HAPT-11-3F7) that specifically recognizes the bovine Haptoglobin heavy chain. Although kit HAPT-11-N uses the same standard/calibrator as kit HAPT-11, we find that HAPT-11-N gives Haptoglobin levels that are 2 to 3.5-fold higher.

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