

HORSE HAPTOGLOBIN ELISA

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

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated up to nine-fold in horse serum as a result of inflammation and infection.^{1,2} Measurement of haptoglobin therefore provides a convenient marker of inflammation and disease in horses.

PRINCIPLE OF THE ASSAY

The horse haptoglobin test kit is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-horse haptoglobin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-horse haptoglobin antibodies for detection. Serum or plasma is denatured and subsequently diluted. The diluted sample is incubated in the microtiter wells for 45 minutes. The microtiter wells are subsequently washed, and 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-labeled antibodies, and TMB Reagent is added and incubated for 20 minutes at room temperature. This results in the development of a blue color. Color development is stopped by the addition of Stop Solution, changing the color to yellow, and optical density is measured spectrophotometrically at 450 nm. The concentration of haptoglobin is proportional to the optical density of the test sample.

MATERIALS AND COMPONENTS

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
- Denaturing buffer; HD25-1, 25 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 or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Plate incubator/shaker
- Plate washer
- Plate reader capable of measuring absorbance at 450 nm.
- Curve fitting software

STORAGE

The unused kit should be stored at 2-8°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 carried out with a complete understanding of the instructions and with adherence to good laboratory practice.
3. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.
4. Laboratory temperature will influence absorbance readings. Our ELISA kits are calibrated using shaking incubators set at 150 rpm and 25°C. Performance of the assay at lower temperatures will result in lower absorbance values

WASH SOLUTION PREPARATION

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.

DILUENT PREPARATION

The diluent is provided as a 10x stock. Prior to use, estimate the final volume of diluent required for your assay and dilute one (1) volume of the 10x stock with nine (9) volumes of distilled or deionized water.

STANDARD PREPARATION

1. The horse 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 100 µg/ml horse haptoglobin stock.
2. Dilute 10 µl of the reconstituted 100 µg/ml horse haptoglobin with 190 µl of denaturing buffer. Incubate at room temperature for at least 10 minutes.
3. Dilute 20 µl of the denatured haptoglobin with 0.98 ml of 1x diluent. This provides the working 100 ng/ml standard.
4. Label 5 polypropylene or glass tubes as 50, 25, 12.5, 6.25 and 3.13 ng/ml.
5. Dispense 500 µl of diluent into the labeled tubes.
6. Prepare a 50 ng/ml standard by diluting and mixing 500 µl of the 100 ng/ml standard with 500 µl of diluent in the tube labeled 50 ng/ml. Similarly prepare the 25, 12.5, 6.25 and 3.13 ng/ml standards by serial dilution.

NOTE: The reconstituted non-denatured haptoglobin stock should be frozen immediately after use. It remains stable in frozen form for at least 6 months at -70°C. Discard the working 100 – 3.13 ng/ml standards after use.

SAMPLE PREPARATION

General Note: Haptoglobin is present in normal horse serum at a concentration of ~ 1 mg/ml. In order to obtain values within the range of the standard curve, we suggest that samples be diluted 32,000-fold using the following procedure for each sample to be tested:

1. Dispense 197.5 µl of denaturing buffer and 997.5 µl of 1x diluent into separate tubes.
2. Pipette and mix 2.5 µl of the serum/plasma sample into the tube containing 197.5 µl of denaturing buffer. This provides an 80-fold diluted, denatured sample. *Please note: the sample must be diluted at least 20-fold in the denaturing buffer.*

3. Allow the samples to incubate in denaturing buffer for at least 10 minutes at room temperature.
4. Mix 2.5 μ l of the 80-fold diluted sample with the 997.5 μ l of 1x diluent in the second tube. This provides a 32,000-fold dilution of the sample.
5. Repeat this procedure for each sample to be tested.

ASSAY PROCEDURE

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

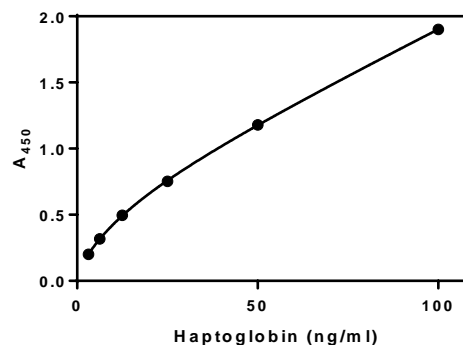
CALCULATION OF RESULTS

1. Using curve fitting software, construct a standard curve by plotting absorbance values of the standards versus concentration.
2. Fit the standard curve to an appropriate model and derive the concentration of the samples (we recommend using a single site, total and nonspecific binding model).
3. Multiply the derived concentration by the dilution factor to determine the actual concentration in the serum sample.
4. If the A_{450} values of samples fall outside the standard curve, samples should be diluted appropriately and re-tested.

TYPICAL STANDARD CURVE

A typical standard curve with absorbance at 450 nm on the Y-axis against haptoglobin concentrations on the X-axis is shown below. This curve is for illustration only.

Haptoglobin (ng/ml)	A_{450}
100	1.901
50	1.180
25	0.753
12.5	0.495
6.25	0.317
3.13	0.201



LIMITATIONS OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of and in accordance with the instructions.
2. The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

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)

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For technical assistance please email us at techsupport@lifediagnosics.com