

HORSE HAPTOGLOBIN ELISA TEST KIT

Life Diagnostics, Inc., Catalog Number: 2410-9

Enzyme Immunoassay for the Determination of Horse Haptoglobin in Serum or Plasma

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 TEST

The horse haptoglobin ELISA 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 30 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:

- Anti-horse haptoglobin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized), containing 100 µg/ml horse haptoglobin when reconstituted as detailed on the vial label
- Wash Buffer (20x stock, 50 ml)
- Denaturing buffer (25 ml)
- 10x Diluent (25 ml)
- TMB Reagent (One-Step) 11 ml
- Stop Solution (1N HCl), 11 ml

Materials required but not provided:

- Precision pipettes and tips
- Distilled or deionized water
- Polypropylene or glass tubes
- Vortex mixer
- Absorbent paper or paper towels
- Micro-Plate incubator/shaker mixing speed of ~150 rpm
- Plate reader with an optical density range of 0-4 at 450nm
- Graph paper (PC graphing software is optional).

STORAGE OF THE KIT

1. For optimum stability store the lyophilized standard at or below -20°C when the ELISA kit is received
2. The remainder of the kit should be stored at 2-8°C and the microtiter strips should be kept in a sealed bag with desiccant to minimize exposure to damp air. Test kits will remain stable for six months from the date of purchase provided that the components are stored as described above.

GENERAL INSTRUCTIONS

All reagents should be allowed to reach room temperature (18-25°C) before use.

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 standard is provided as a lyophilized stock. Add the volume of distilled or de-ionized 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 labelled 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 haptoglobin standard 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 μl of the 80 fold diluted sample with the 997.5 μl of 1 x 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
6. If the A_{450} values of samples fall outside the 100 – 3.13 ng/ml standard range when tested at a dilution of 32,000, samples should be re-diluted appropriately and re-tested. In the event that alternative dilutions are to be tested, prepare them by diluting from the denatured sample (prepared as described above) into 1x diluent. A minimum final dilution of 1000 fold must be achieved, i.e., at least a 50 fold dilution from the 20 fold diluted denatured sample into 1x diluent.

ASSAY PROCEDURE

1. Secure the desired number of coated wells in the holder.
2. Dispense 100 μl of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
4. Remove the incubation mixture using either a plate washer or by flicking plate contents into an appropriate bio-waste container.
5. Wash and empty the microtiter wells 5 times with 1x wash solution. This may be performed using either a plate washer (400 μl /well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
6. Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
7. Add 100 μl of enzyme conjugate reagent into each well.
8. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 30 minutes.
9. Wash as detailed in 4 to 5 above.
10. Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
11. Dispense 100 μl of TMB Reagent into each well.
12. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
13. Stop the reaction by adding 100 μl of Stop Solution to each well.
14. Gently mix. *It is important to make sure that all the blue color changes to yellow.*
15. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

CALCULATION OF RESULTS

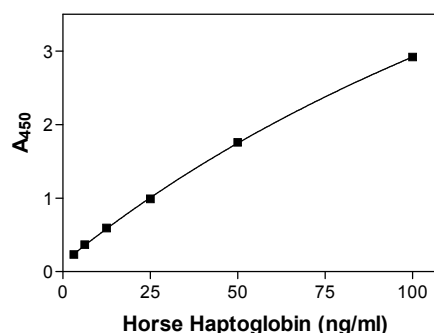
1. Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
3. Using the mean absorbance value for each sample, determine the corresponding concentration of haptoglobin in ng/ml from the standard curve.
4. Multiply the derived concentrations by the dilution factor to determine the actual concentration of haptoglobin in the serum/plasma sample.
5. PC graphing software may be used for the above steps.

TYPICAL STANDARD CURVE

A typical standard curve with optical density readings at 450nm on the Y axis against haptoglobin concentrations on the X axis is shown below. This curve is for the purpose of illustration only and should not be used to calculate unknowns. Each user should obtain his or her data and standard curve in each experiment.

Haptoglobin (ng/ml)	Absorbance (450 nm)
100	2.919
50	1.756
25	0.988
12.5	0.590
6.25	0.365
3.13	0.233

Typical Horse Haptoglobin Standard Curve



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)