

DOG HAPTOGLOBIN ELISA TEST KIT

Life Diagnostics, Inc., Catalog Number: 2410-3

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

INTRODUCTION

Haptoglobin is an acute phase protein that is elevated in dog serum as a result of inflammation¹. Serum levels also increase approximately two fold during pregnancy². Measurement of haptoglobin provides a convenient marker of inflammation/disease in dogs.

PRINCIPLE OF THE TEST

The dog haptoglobin ELISA is based on a solid phase enzyme-linked immunosorbent assay (ELISA). The assay uses affinity purified anti-dog haptoglobin antibodies for solid phase (microtiter wells) immobilization and horseradish peroxidase (HRP) conjugated anti-dog haptoglobin antibodies for detection. The test sample is diluted and 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-dog haptoglobin antibody coated microtiter plate with 96 wells (provided as 12 detachable strips of 8)
- Enzyme Conjugate Reagent, 11 ml
- Reference standard (lyophilized), containing 2 µg/ml dog haptoglobin when reconstituted as detailed on the vial label
- Wash Buffer (20x stock, 50 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 TEST KIT

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

1. All reagents should be allowed to reach room temperature (18-25°C) before use.
2. Serum or plasma samples should be diluted ~100,000 fold with 1x diluent in order to obtain values within the standard range.

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 dog 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 2 µg/ml dog haptoglobin stock (*the reconstituted standard remains stable for at least 1 day at 2-8°C but should be aliquoted and frozen at -20°C after reconstitution if use beyond this time is intended*).
2. Label 8 polypropylene or glass tubes as 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.95 and 0 ng/ml.
3. Dispense 937.5 µl of diluent into the tube labeled 125 ng/ml and 300 µl of diluent into the remaining tubes.
4. Pipette 62.5 µl of the 2 µg/ml haptoglobin standard into the tube labeled 125 ng/ml and mix. This provides the working 125 ng/ml haptoglobin standard.
5. Prepare a 62.5 ng/ml standard by diluting and mixing 300 µl of the 125 ng/ml standard with 300 µl of diluent in the tube labeled 62.5 ng/ml. Similarly prepare the 31.25, 15.6, 7.8, 3.9 and 1.95 ng/ml standards by serial dilution.

SAMPLE PREPARATION

General Note: Haptoglobin is present in normal dog 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 100,000 fold using the following procedure for each sample to be tested:

1. Dispense 998 µl and 497.5 µl of 1x diluent into separate tubes.

- Pipette and mix 2 μl of the serum/plasma sample into the tube containing 998 μl of diluent. This provides a 500 fold diluted sample.
- Mix 2.5 μl of the 500 fold diluted sample with the 497.5 μl of diluent in the second tube. This provides a 100,000 fold dilution of the sample.
- Repeat this procedure for each sample to be tested

ASSAY PROCEDURE

- Secure the desired number of coated wells in the holder.
- Dispense 100 μl of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.
- Remove the incubation mixture using either a plate washer or by flicking plate contents into an appropriate Bio-waste container.
- Wash and empty the microtiter wells 5 times with 1x wash solution. This may be performed using either a plate washer (350 μl /well) or with a squirt bottle. The entire wash procedure should be performed as quickly as possible.
- Strike the wells sharply onto absorbent paper or paper towels to remove all residual droplets.
- Add 100 μl of enzyme conjugate reagent into each well.
- Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 30 minutes.
- Wash as detailed in 4 to 5 above.
- Strike the wells sharply onto absorbent paper or paper towels to remove residual droplets.
- Dispense 100 μl of TMB Reagent into each well.
- Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.
- Stop the reaction by adding 100 μl of Stop Solution to each well.
- Gently mix. *It is important to make sure that all the blue color changes to yellow.*
- Read the optical density at 450 nm with a microtiter plate reader within 15 minutes.

CALCULATION OF RESULTS

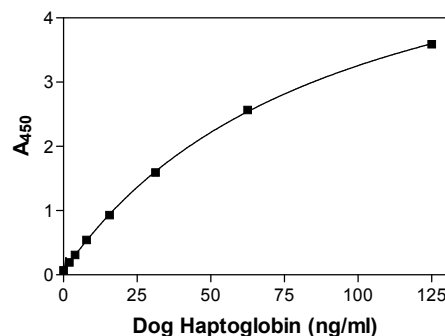
- Calculate the average absorbance values (A_{450}) for each set of reference standards, and samples.
- Construct a standard curve by plotting the mean absorbance obtained from each reference standard against its concentration in ng/ml on linear graph paper, with absorbance values on the vertical or Y-axis and concentrations on the horizontal or X-axis.
- Using the mean absorbance value for each sample, determine the corresponding concentration of haptoglobin in ng/ml from the standard curve.
- Multiply the derived concentrations by the dilution factor to determine the actual concentration of haptoglobin in the serum/plasma sample.
- PC graphing software may be used for the above steps.
- If the A_{450} values of samples fall outside the standard curve when tested at a dilution of 100,000. Samples should be diluted appropriately and re-tested.

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)
125	3.590
62.5	2.567
31.25	1.592
15.63	0.928
7.81	0.541
3.91	0.310
1.95	0.192
0	0.068

Representative Dog Haptoglobin Standard Curve



LIMITATIONS OF THE PROCEDURE

- Do not use grossly hemolyzed samples. Serum hemoglobin concentrations of 0.1 mg/ml have no effect but concentrations of 1 mg/ml cause an approximate 20% decrease in apparent haptoglobin levels.
- 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.
- The wash procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

REFERENCES

- Solter, PF. et. al. Haptoglobin and ceruloplasmin as determinants of inflammation in dogs. *Am J Vet Res.* 52:1738-42 (1991)
- Vannucchi, CI, Mirandola, RM and Oliviera, CM. Acute-phase protein profile during gestation and diestrous: proposal for an early pregnancy test in bitches. *Animal Reproduction Science.* 74: 87-99 (2002)