Canine Haptoglobin ELISA Quantitation Kit

Manual

Catalog number: 40-374-130028

Immunoperoxidase Assay for Determination of Haptoglobin in Dog Sera/Plasma.
This kit is for research use only, and is not for use in diagnostic procedures.

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**INTENDED USE**

The HAPTOGLOBIN test kits are a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring HAPTOGLOBIN in serum or plasma of Dogs.

**INTRODUCTION**

Acute phase proteins are plasma proteins which increase in concentration following infection, inflammation or trauma. The first acute phase protein to be recognized was discovered in humans by Tillet and Frances in 1930. Haptoglobin (Hp) is a heterogeneous plasma protein mostly synthesized in the liver. The haptoglobin monomer consist of two heavy chains, beta chains (40 kD) and two light chains, alpha chains, alpha 1 (9 kD) and alpha 2 (16 kD) that are linked disulfide bonds. The three major haptoglobin types are; Hp1-1 which is monomeric (98kD), Hp1-2 is polymeric at about 200 kD, and Hp2-2 at about 400 kD. The haptoglobin level in serum rise quickly following acute tissue damage within 24 to 48 hours and also falls very rapidly once the stimulus is removed. In fact, Hp level are decreased in hemolytic anemia. Hp has a high affinity for hemoglobin (Hb) and its function appears to be to prevent loss of Hb in urine which would lead to loss of iron. Investigations over the past few years have shown that quantification of Hp in plasma or serum can provide valuable diagnostic information in the detection, prognosis, and monitoring of disease not only in humans, but in companion animals and farm herds as well.

**PRINCIPLE OF THE TEST**

The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the HAPTOGLOBIN present in serum sample reacts with the anti-HAPTOGLOBIN antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound serum proteins by washing, anti-Hp antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes with the previously bound serum Hp. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of Hp in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of Hp in the test sample. The quantity of Hp in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for serum dilution.
Anti-Hp Antibodies Bound To Solid Phase  
Control and Patient Serum Samples Added  
Hp-Anti-Hp Complexes Formed  
Unbound Serum Proteins Removed  
Anti-Hp-HRP Conjugate Added  
Anti-Hp-HRP * Hp * Anti-Hp Complexes Formed  
Unbound Anti-Hp-HRP Removed  
Chromogenic Substrate Added  
Determine Bound Enzyme Activity

Figure 1.

REAGENTS

(Quantities sufficient for 96 determinations)

1. DILUENT
One bottle containing 50 ml of a 5X concentrated phosphate buffered saline (PBS) solution containing bovine serum albumin, 0.25% Tween, and 0.1% Proclin 300 as a preservative.

2. WASH SOLUTION CONCENTRATE
One bottle containing 50 ml of a 20X concentrated phosphate buffered saline (PBS) solution containing 0.5% Tween.

3. ENZYME-ANTIBODY CONJUGATE 100X
One vial containing 200 μL of affinity purified anti- CANINE HAPTOGLOBIN antibody conjugated with horseradish peroxidase in a stabilizing buffer.

4. CHROMOGEN-SUBSTRATE SOLUTION
One vial containing 12 mL of 3,3′,5,5′-tetramethybenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3.

5. STOP SOLUTION
One vial containing 12 ml 0.3 M sulfuric acid.

WARNING: Avoid contact with skin.

6. ANTI-CANINE HAPTOGLOBIN ELISA MICRO PLATE
Twelve removable eight (8) well micro well strips in well holder frame. Each well is coated with affinity purified anti-CANINE Haptoglobin.

7. CANINE HAPTOGLOBIN STANDARDS
One vial containing a lyophilized CANINE Haptoglobin calibrator.

FOR IN VITRO USE ONLY

REAGENT PREPARATION

1. DILUENT
The Wash Solution supplied is a 5X Concentrate and must be diluted 1:5 with distilled or deionized water.

2. WASH SOLUTION CONCENTRATE
The Wash Solution supplied is a 20X Concentrate and must be diluted 1:20 with distilled or deionized water. Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.

3. ENZYME-ANTIBODY CONJUGATE
The required amount of working conjugate solution for each microtitre plate is prepared by adding 100 μL Enzyme-Antibody Conjugate to 10 mL of Diluent. Mix uniformly, but gently. Avoid foaming.

4. CHROMOGEN-SUBSTRATE SOLUTION
Ready to use as supplied.

5. STOP SOLUTION
Ready to use as supplied.

6. ANTI-CANINE HAPTOGLOBIN ELISA MICRO PLATE
Ready to use as supplied.

7. CANINE HAPTOGLOBIN STANDARDS
Add 1.0 ml of distilled or de-ionized water to the Canine Haptoglobin Calibrator and mix gently until dissolved. The calibrator is now at a concentration of 10.5 μg/ml (the reconstituted calibrator should be aliquoted and frozen if future use is intended). Canine Haptoglobin standards need to be prepared immediately prior to use (see chart below). Mix well between each step. Avoid foaming.

<table>
<thead>
<tr>
<th>Standard</th>
<th>ng/mL</th>
<th>Volume added to 1x Diluent</th>
<th>Volume of 1x Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>125</td>
<td>6 μL Canine Hpt Calibrator</td>
<td>500 μL</td>
</tr>
<tr>
<td>2</td>
<td>62.5</td>
<td>250 μL standard 1</td>
<td>250 μL</td>
</tr>
<tr>
<td>3</td>
<td>31.25</td>
<td>250 μL standard 2</td>
<td>250 μL</td>
</tr>
<tr>
<td>4</td>
<td>15.6</td>
<td>250 μL standard 3</td>
<td>250 μL</td>
</tr>
<tr>
<td>5</td>
<td>7.8</td>
<td>250 μL standard 4</td>
<td>250 μL</td>
</tr>
<tr>
<td>6</td>
<td>3.9</td>
<td>250 μL standard 5</td>
<td>250 μL</td>
</tr>
<tr>
<td>7</td>
<td>1.95</td>
<td>250 μL standard 6</td>
<td>250 μL</td>
</tr>
</tbody>
</table>
**STORAGE AND STABILITY**

The expiration date for the package is stated on the box label.

1. **DILUENT**
The 5X Diluent Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4-8°C.

2. **WASH SOLUTION**
The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions can be stored at room temperature (16-25°C) or at 4-8°C.

3. **ENZYME-ANTIBODY CONJUGATE**
Undiluted horseradish peroxidase anti-Hp conjugate should be stored at 4-8°C and diluted immediately prior to use. The working conjugate solution is stable for one day.

4. **CHROMOGEN-SUBSTRATE SOLUTION**
The Substrate Solution should be stored at 4-8°C and is stable until the expiration date.

5. **STOP SOLUTION**
The Stop Solution should be stored at 4-8°C and is stable until the expiration date.

6. **ANTI-CANINE HAPTOGLOBIN ELISA MICRO PLATE**
Anti-CANINE Hp coated wells are stable until the expiration date, and should be stored at 4-8°C in the sealed foil pouch.

7. **CANINE HAPTOGLOBIN STANDARDS**
The lyophilized Canine Haptoglobin Calibrator should be stored at 4°C or frozen until reconstituted. The reconstituted calibrator should be aliquoted and stored frozen (avoid multiple freeze-thaw cycles). The working standard solutions should be prepared immediately prior to use and are stable for 1 day.

**INDICATIONS OF INSTABILITY**

If the test is performing correctly, the results observed with the standard solutions should be within 20% of the expected values.

**SPECIMEN COLLECTION AND PREPARATION**

Blood should be collected by venipuncture and the serum separated from the cells, after clot formation, by centrifugation. Specimens may be shipped at room temperature and then stored refrigerated at 2-8°C if testing is to take place within one week after collection. If testing is to take place later than one week, specimens should be stored at -20°C. Avoid repeated freeze-thaw cycles.

1. **Precautions**
For any sample that might contain pathogens, care must be taken to prevent contact with open wounds.

2. **Additives and Preservatives**
No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.
3. Known interfering substances

Azide and thimerosal at concentrations higher than 0.1% inhibits the enzyme reaction.

**MATERIAL PROVIDED**
See "REAGENTS"

**MATERIALS REQUIRED BUT NOT PROVIDED**

- Precision pipette (2 μL to 200 μL) for making and dispensing dilutions
- Test tubes
- Microtitre washer/aspirator
- Distilled or Deionized H2O
- Microtitre Plate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Vortex mixer

**ASSAY PROCEDURE**

**DILUTION OF SERUM SAMPLES**

The assay for quantification of Hp in serum requires that each test sample be diluted before use. For a single step determination a dilution of serum at 1:10,000 is appropriate for most samples. For absolute quantification, samples that yield results outside the range of the standard curve, a lesser or greater dilution might be required.

1. To prepare a 1:10,000 dilution of sample, transfer 5 μL of serum sample to 495 μL of diluent. This gives you a 1:100 dilution. Next, dilute the 1:100 sample by transferring 5 μL, to 495 μL of diluent. You now have a 1:10,000 dilution of your sample. Mix thoroughly at each stage.

**PROCEDURE**

Bring all reagents to room temperature before use.

1. Add 100 μL of Diluent to each of the wells in 1A & 2A. These will serve for an evaluation of the background associated with the assay.

2. Pipette 100 μL of
   - Standard 1 (125 ng/ml) into wells 1B & 2B
   - Standard 2 (62.5 ng/ml) into wells 1C & 2C
   - Standard 3 (31.25 ng/ml) into wells 1D & 2D
   - Standard 4 (15.6 ng/ml) into wells 1E & 2E
   - Standard 5 (7.8 ng/ml) into wells 1F & 2F
   - Standard 6 (3.9 ng/ml) into wells 1G & 2G
   - Standard 7 (1.95 ng/ml) into wells 1H & 2H

3. Pipette 100 μL of serum sample (test sample 1) into wells 3A & 4A. The next sample goes in wells 3B & 4B, the next in 3C & 4C and so on.
4. Incubate the micro titer plate at 22°C (room temperature) for fifteen (15 ± 2) minutes. Keep plate level during incubation.

5. Following incubation, aspirate the contents of the wells.

6. Completely fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. If washing manually: completely fill wells with wash buffer, invert the plate then pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of four washes.

7. Pipette 100 μL of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at 22°C (room temperature) for fifteen (15 ± 2) minutes.

8. Wash and blot the wells as described in Steps 5/6.

9. Pipette 100 μL of TMB Substrate Solution into each well.

10. Incubate at room temperature for precisely ten (10) minutes.

11. After ten minutes, add 100 μL of Stop Solution to each well.

12. Determine the absorbance (450 nm) of the contents of each well. Calibrate the plate reader to air.

**STABILITY OF THE FINAL REACTION MIXTURE**

The absorbance of the final reaction mixture can be measured up to 2 hours after the addition of the Stop Solution. However, good laboratory practice dictates that the measurement be made as soon as possible.

**RESULTS**

1. Subtract the average background value from the test values for each sample.

2. Using the results observed for the standards construct a Standard Curve. The appropriate curve fit is that of a four-parameter logistics curve. A second order polynomial (quadratic) or other curve fits may also be used.

3. Interpolate test sample values from standard curve. Correct for sera dilution factor to arrive at the haptoglobin concentration in original sample.

**QUALITY CONTROL**

In accord with good laboratory practice, the Assays for specific Hp require meticulous quality control. Each laboratory should use routine quality control procedures to establish inter- and intra-assay precision and performance characteristics.
LIMITATION OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the information contained in the package insert instructions and with adherence to good laboratory practice.

2. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or deionized water, and accuracy of reagent and sample pipettings.

REFERENCES


TROUBLESHOOTING

The following are some common problems encountered with the use of ELISA kits, and some of the causes of these problems.

1. **Problem: Low absorbance**
   - Incorrect dilutions or pipetting errors.
   - Improper incubation times.
   - Improper mixing of the TMB substrate. Each component is mixed in equal parts.
   - Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB, 490 nm for OPD, or 405 nm for ABTS.
   - Kit materials or reagents are contaminated or expired.
   - Incorrect reagents used.

2. **Problem: High Absorbance**
   - Cross contamination from other samples or positive control.
   - Incorrect dilutions or pipetting errors.
   - Improper washing.
   - Wrong filter on microtiter reader.
   - Contaminated buffers or enzyme substrate.
   - Improper incubation times.
   - Kit materials or reagents are contaminated or expired.

3. **Problem: Poor Duplicates**
   - Poor mixing of specimens.
   - Incorrect dilutions or pipetting errors.
   - Technical error.
   - Inconsistency in following ELISA protocol.
   - Inefficient washing.

4. **Problem: All wells are positive**
   - Contaminated buffers or enzyme substrate.
5. Problem: All wells are negative
   □ Procedure not followed correctly.
   □ Contaminated buffers or enzyme substrate.
   □ Contaminated conjugate.
   □ Kit materials or reagents are contaminated or expired.

The above information is believed to be correct but does not purport to be all-inclusive and is intended to be used only as a guide. GenWay Biotech, Inc. shall not be liable or responsible in any way for use of either this information or the material supplied. Disposal of hazardous material may be subject to federal, state or local laws or regulations.