KAMIYA BIOMEDICAL COMPANY

Horse Haptoglobin ELISA

For the quantitative determination of haptoglobin in horse biological samples

Cat. No. KT-488

For Research Use Only.

PRODUCT INFORMATION

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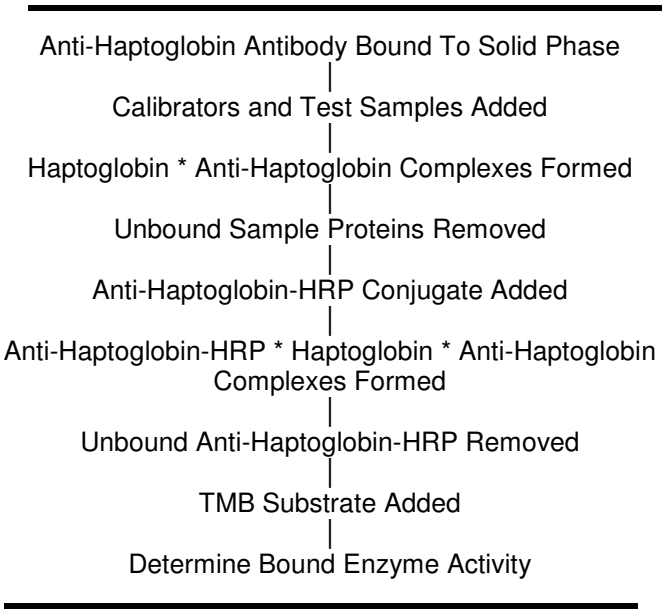
INTENDED USE
The Horse Haptoglobin ELISA is a highly sensitive two-site enzyme-linked immunoassay (ELISA) for the quantitative determination of haptoglobin in horse biological samples. For research use only.

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

PRINCIPLE
The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the haptoglobin present in samples reacts with the anti-haptoglobin antibodies which have been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-haptoglobin antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes with the previously bound haptoglobin. 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 haptoglobin in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of haptoglobin in the test sample. The quantity of haptoglobin in the test sample can be interpolated from the calibration curve constructed from the calibrators, and corrected for sample dilution.

Figure 1.



COMPONENTS

1. Diluent Concentrate
   One bottle containing 50 mL of a 5X concentrated diluent running buffer.

2. Wash Solution Concentrate
   One bottle containing 50 mL of a 20X concentrated wash solution.

3. Enzyme-Antibody Conjugate Concentrate
   One vial containing 150 µL of a 100X concentrated affinity-purified anti-horse haptoglobin antibody conjugated with HRP in stabilizing buffer.

4. TMB Substrate Solution
   One vial containing 12 mL of TMB and hydrogen peroxide in citric acid buffer at pH 3.3.

5. Stop Solution
   One vial containing 12 mL of 0.3 M sulfuric acid.
   WARNING: Avoid contact with skin.

6. Microtiter Plate
   Twelve removable eight-well strips in well holder frame. Wells are coated with affinity-purified anti-horse haptoglobin.

7. Horse Haptoglobin Calibrator
   One vial containing a lyophilized Horse Haptoglobin Calibrator.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipette (2 µL to 200 µL) for making and dispensing dilutions
- Test tubes
- Microplate washer/aspirator
- Distilled or de-ionized H_2O
- Microplate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer

PRECAUTIONS

1. Read the instructions carefully before beginning the assay.
2. This kit is for research use only.
3. Great care has been taken to ensure the quality and reliability of this product. However, it is possible that in certain cases, unusual results may be obtained due to high levels of interfering factors.
4. No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.
5. Azide and thimerosal at concentrations higher than 0.1% inhibit the enzyme reaction.
6. Other precautions:
   - Do not interchange kit components from different lots.
   - Do not use kit components beyond the expiration date.
   - Protect reagents from direct sunlight.
   - Do not pipette by mouth.
   - Do not eat, drink, smoke or apply cosmetics where reagents are used.
   - Avoid all contact with the reagents by using gloves.
   - Stop solution contains diluted sulfuric acid. Irritation to eyes and skin is possible. Flush with water after contact.

REAGENT PREPARATION

1. Diluent Concentrate
   The Diluent solution supplied is a 5X concentrate and must be diluted 1:5 with distilled or de-ionized water.

2. Wash Solution Concentrate
   The Wash Solution supplied is a 20X concentrate and must be diluted 1:20 with distilled or de-ionized 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 Concentrate
   Calculate the required amount of working conjugate solution for each microtiter plate test strip by adding 10 µL Enzyme-Antibody Conjugate to 990 µL of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently. Avoid foaming.

4. TMB Substrate Solution
   Ready to use as supplied.

5. Stop Solution
   Ready to use as supplied.

6. Microtiter Plate
   Ready to use as supplied. Unseal microtiter pouch and remove plate from pouch. Remove all strips and wells that will not be used in the assay and place back in pouch and re-seal.

7. Horse Haptoglobin Calibrator
   Add 1.0 mL of distilled or de-ionized water to the lyophilized Horse Haptoglobin calibrator and mix gently until dissolved. The calibrator is now at a concentration of 10.86 µg/mL **(the reconstituted calibrator should be aliquoted and frozen if future use is intended). Horse Haptoglobin calibrators need to be prepared immediately prior to use (see chart below).** Mix well between each step. Avoid foaming.

Calibrator	Concentration (ng/mL)	Calibrator Volume added to 1X Diluent →	Volume of 1X Diluent
6	600	50 µL Horse Haptoglobin Calibrator	855 µL
5	300	300 µL Calibrator 6	300 µL
4	150	300 µL Calibrator 5	300 µL
3	75	300 µL Calibrator 4	300 µL
2	37.5	300 µL Calibrator 3	300 µL
1	18.75	300 µL Calibrator 2	300 µL
0	0		600 µL

STORAGE AND STABILITY

1. Complete Kit
   The expiration date for the kit is stated on the outer label. The recommended storage temperature is 4°C. **Note: See long term storage recommendations below for the Horse Haptoglobin Calibrator.**

2. 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°C.

3. 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 (RT, 16-25°C) or at 4°C.

4. Enzyme-Antibody Conjugate
   Undiluted anti-haptoglobin-HRP conjugate should be stored at 4°C and **diluted immediately prior to use**. The working conjugate solution is stable for up to 1 hour when stored in the dark.

5. TMB Substrate Solution
   The TMB Substrate Solution should be stored at 4°C and is stable until the expiration date.

6. Stop Solution
   The Stop Solution should be stored at 4°C and is stable until the expiration date.

7. Microtiter Plate
   Anti-horse haptoglobin coated wells are stable until the expiration date, and should be stored at 4°C in the sealed foil pouch with desiccant pack.

8. Horse Haptoglobin Calibrator
   The lyophilized Horse Haptoglobin calibrator should be stored at 4°C or frozen until reconstituted. The reconstituted calibrator should be aliquoted out and stored frozen (avoid multiple freeze-thaw cycles). The working calibrator solutions should be prepared immediately prior to use and are stable for up to 8 hours.

INDICATIONS OF INSTABILITY

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

SPECIMEN COLLECTION AND HANDLING

Blood should be collected by venipuncture and the serum separated from the cells, after clot formation, by centrifugation. For plasma samples, blood should be collected into a container with an anticoagulant and then centrifuged. Care should be taken to minimize hemolysis, excessive hemolysis can impact your results. Assay immediately or aliquot and store samples at -20°C. Avoid repeated freezing/thawing.

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds. No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

ASSAY PROTOCOL

Dilution of Samples

Due to the high sensitive nature of the assay each sample should be diluted before use for a normal assay. A 1:10,000 dilution of serum or plasma is appropriate for most samples. For absolute quantification of samples that yield results outside the range of the calibration curve, a lesser or greater dilution might be required. **If unsure of sample level, a serial dilution with one or two representative samples before running the entire plate is highly recommended.**

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

Procedure

Bring all reagents to RT before use.

1. Pipette 100 µL of
   Calibrator 0 (0.0 ng/mL) in duplicate
   Calibrator 1 (18.75 ng/mL) in duplicate
   Calibrator 2 (37.5 ng/mL) in duplicate
   Calibrator 3 (75 ng/mL) in duplicate
   Calibrator 4 (150 ng/mL) in duplicate
   Calibrator 5 (300 ng/mL) in duplicate
   Calibrator 6 (600 ng/mL) in duplicate

2. Pipette 100 µL of diluted sample in duplicate into pre-designated wells.

3. Incubate the Microtiter Plate at 22°C (RT) for thirty (30 ± 2) minutes. Keep plate level and covered during incubation.

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

5. 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 diluted Wash Solution, invert the plate and pour/shake out the

contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual Wash Solution. Repeat three times for a total of four washes.

6. Pipette 100 µL of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at 22°C (RT) for thirty (30 ± 2) minutes. Keep plate covered in the dark and level during incubation.

7. Wash and blot the wells as described in Steps 4 and 5.

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

9. Incubate in the dark at RT for precisely ten (10) minutes.

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

11. Determine the absorbance at 450 nm of the contents of each well. Calibrate the plate reader to manufacturer's specifications.

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 calibrators construct a calibration curve. The appropriate curve fit is that of a four-parameter logistics curve, although a second order polynomial (quadratic) or other curve fits may also be used.

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

PERFORMANCE CHARACTERISTICS

In accord with good laboratory practice, the assays for specific haptoglobin 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 de-ionized water, washing technique, incubation time or temperature, and accuracy of reagent and sample pipettings.

FOR RESEARCH USE ONLY

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