

KAMIYA BIOMEDICAL COMPANY

Rabbit Haptoglobin ELISA

**For the quantitative determination of haptoglobin
in rabbit biological fluid**

Cat. No. KT-352

For Research Use Only.

PRODUCT INFORMATION

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INTENDED USE

The Rabbit Haptoglobin ELISA is a highly sensitive two-site enzyme-linked immunoassay (ELISA) for the quantitative determination of haptoglobin in rabbit biological fluid. For research use only.

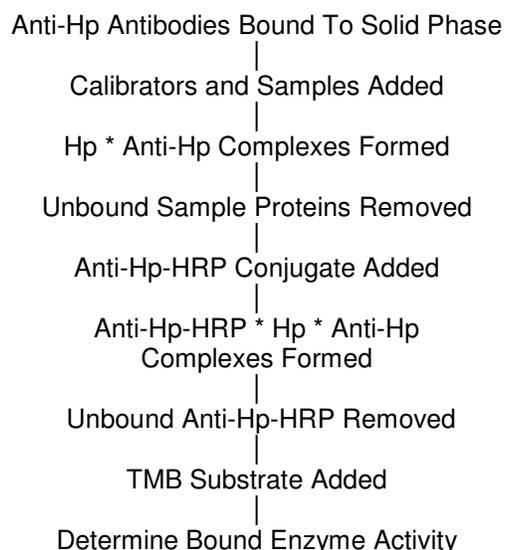
INTRODUCTION

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 kD), and two light chains, alpha chains, alpha 1 (9 kD) and alpha 2 (16 kD) that are linked by disulfide bonds. The three major haptoglobin types are; Hp1-1, which is monomeric (98 kD), Hp1-2 is polymeric at about 200 kD, and Hp2-2 at about 400 kD. The levels in serum rise quickly following acute tissue damage within 24 to 48 hours and also fall 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-Hp antibodies, which have been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-Hp 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 μ L of a 100X concentrated affinity purified anti-rabbit haptoglobin antibody conjugated with HRP in a 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 micro strips in well holder frame. Each well is coated with affinity purified anti-rabbit haptoglobin.
7. Rabbit Haptoglobin Calibrator
One vial containing a lyophilized Rabbit Haptoglobin Calibrator.
8. Positive Control
One vial containing 50 μ L of serum with 0.1% sodium azide. See the Control Certificate for the concentration.

MATERIALS REQUIRED BUT NOT PROVIDED

- Test tubes
- Precision pipette (2 μ L to 200 μ L) for making and dispensing dilutions
- Microtiter washer/aspirator
- Distilled or De-ionized H₂O
- Microtitre Plate 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. Preservatives
 - Positive Control contains 0.1% sodium azide.
5. No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.
6. Azide and thimerosal at concentrations higher than 0.1% inhibit the enzyme reaction.
7. 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 along with desiccant.

7. Rabbit Haptoglobin Calibrator

Add 1.0 mL of distilled or de-ionized water to the lyophilized Rabbit Haptoglobin Calibrator and mix gently until dissolved. The calibrator is now at a concentration of 24.8 µg/mL (the reconstituted calibrator should be aliquoted and frozen if future use is intended). Prepare the Rabbit Haptoglobin Calibrators immediately prior to use according to the table below. Mix well between each step. Avoid foaming.

Calibrator	Concentration (ng/mL)	Calibrator Volume added to 1X Diluent	→ Volume of 1X Diluent
6	200	5 µL Rabbit Haptoglobin Calibrator	615 µL
5	100	0.3 mL Calibrator 6	0.3 mL
4	50	0.3 mL Calibrator 5	0.3 mL
3	25	0.3 mL Calibrator 4	0.3 mL
2	12.5	0.3 mL Calibrator 3	0.3 mL
1	6.25	0.3 mL Calibrator 2	0.3 mL
0	0		0.6 mL

8. Positive Control

The concentration and recommended dilution are provided on the Control Certificate. Before use, briefly centrifuge the Positive Control to allow all of the liquid to collect in the bottom of the vial.

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 Rabbit Haptoglobin Calibrator and Positive Control.**

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-Hp-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-rabbit haptoglobin coated wells are stable until the expiration date and should be stored at 4°C in the sealed foil pouch with a desiccant pack.

8. Rabbit Haptoglobin Calibrator

The lyophilized Rabbit 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 calibrator solutions should be prepared immediately prior to use and are stable for up to 8 hours.

9. Positive Control

For storage longer than 7 days keep frozen until the expiration date. Storage less than 7 days can be at 4°C. Avoid multiple freeze/thaw cycles.

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 1:4,000 dilution is appropriate for most serum or plasma 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 1:4,000 dilution of sample, transfer 5 µL of sample to 495 µL of 1X Diluent. This gives you a 1:100 dilution. Next, dilute the 1:100 sample by transferring 10 µL, to 390 µL of 1X Diluent. You now have a 1:4,000 dilution of your sample. Mix thoroughly at each stage.

Procedure

1. Bring all reagents to RT before use.
2. Pipette 100 µL of
 - Calibrator 0 (0.0 ng/mL) in duplicate
 - Calibrator 1 (6.25 ng/mL) in duplicate
 - Calibrator 2 (12.5 ng/mL) in duplicate
 - Calibrator 3 (25 ng/mL) in duplicate
 - Calibrator 4 (50 ng/mL) in duplicate
 - Calibrator 5 (100 ng/mL) in duplicate
 - Calibrator 6 (200 ng/mL) in duplicate
3. Pipette 100 µL of diluted Positive Control (in duplicate) into pre designated wells.
4. Pipette 100 µL of diluted sample (in duplicate) into pre designated wells.
5. Incubate the Microtiter Plate at 22°C (RT) for sixty (60 ± 2) minutes. Keep plate covered and level during incubation.

6. Following incubation, aspirate the contents of the wells.
7. 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.
8. Pipette 100 μ L of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at 22°C (RT) for twenty (20 ± 2) minutes. Keep plate covered in the dark and level during incubation.
9. Wash and blot the wells as described in Steps 6 and 7.
10. Pipette 100 μ L of TMB Substrate Solution into each well.
11. Incubate in the dark at RT for precisely ten (10) minutes.
12. After ten minutes, add 100 μ L of Stop Solution to each well.
13. 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 two 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. A second order polynomial (quadratic) or other curve fits may also be used.
3. Interpolate test sample values from the calibration curve. Correct for sample dilution factor to arrive at haptoglobin concentration in original sample.

QUALITY CONTROL

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, and accuracy of reagent and sample pipettings, washing technique, incubation time or temperature.

FOR RESEARCH USE ONLY

KAMIYA BIOMEDICAL COMPANY
12779 Gateway Drive, Seattle, WA 98168
Tel: (206) 575-8068 Fax: (206) 575-8094
Email: LifeScience@k-assay.com
www.k-assay.com