

**KAMIYA BIOMEDICAL COMPANY**

# **Bovine Haptoglobin ELISA**

**For the quantitative determination of  
Haptoglobin in bovine biological samples**

**Cat. No. KT-191**

**For research use only.**

## PRODUCT INFORMATION

### **Bovine Haptoglobin ELISA** Cat. No. KT-191

#### **INTENDED USE**

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

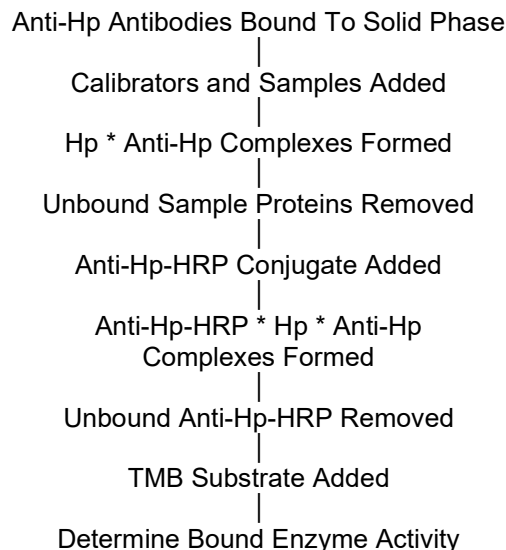
#### **INTRODUCTION**

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 in the liver. The haptoglobin monomer consists of two heavy chains, beta chains (40 kDa) and two light 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 at 98 kDa, Hp1-2 is polymeric at about 200 kDa and Hp2-2 at about 400 kDa. The haptoglobin levels in serum rise quickly following acute tissue damage within 24 to 48 hours and fall quickly 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 Hb in urine, which would lead to loss of iron. Investigations over the past few years have shown that quantification of haptoglobin in plasma and 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 the sample 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. This HRP-conjugated antibody forms a complex 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 is proportional to 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  $\mu$ L of a 100X concentrated affinity-purified anti-bovine haptoglobin antibody conjugated with HRP in a stabilizing buffer.
4. TMB Substrate Solution  
One bottle containing 12 mL of TMB and hydrogen peroxide in citric acid buffer at pH 3.3.
5. Stop Solution  
One bottle 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-bovine haptoglobin.
7. Bovine Haptoglobin Calibrator  
One vial containing a lyophilized Bovine Haptoglobin Calibrator.

## MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipettes (2  $\mu$ L to 200  $\mu$ L) for making and dispensing dilutions
- Test tubes
- Squirt bottle or Microplate washer/aspirator
- Distilled or de-ionized H<sub>2</sub>O
- Microplate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Centrifuge for sample collection
- Anticoagulant for plasma collection

## 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. Azide and thimerosal at concentrations higher than 0.1% inhibit the enzyme reaction.
5. 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  $\mu\text{L}$  Enzyme-Antibody Conjugate to 990  $\mu\text{L}$  of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently. Avoid foaming. Dilute immediately before use and protect from light.

## 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. Bovine Haptoglobin Calibrator

Add 1.0 mL of distilled or de-ionized water to the lyophilized Bovine Haptoglobin Calibrator and mix gently until dissolved. The calibrator is now at a concentration of 10.143  $\mu\text{g}/\text{mL}$  (**the reconstituted calibrator should be aliquoted and frozen if future use is intended**). **Bovine Haptoglobin Calibrators need to be prepared immediately prior to use (see the following chart)**. Mix well between each step. Avoid foaming.

Calibrator	Concentration (ng/mL)	Calibrator Volume added to 1X Diluent	Volume of 1X Diluent
7	1,000	70 $\mu\text{L}$ Bovine Hp Calibrator	640 $\mu\text{L}$
6	500	300 $\mu\text{L}$ Calibrator 7	300 $\mu\text{L}$
5	250	300 $\mu\text{L}$ Calibrator 6	300 $\mu\text{L}$
4	125	300 $\mu\text{L}$ Calibrator 5	300 $\mu\text{L}$
3	62.5	300 $\mu\text{L}$ Calibrator 4	300 $\mu\text{L}$
2	31.25	300 $\mu\text{L}$ Calibrator 3	300 $\mu\text{L}$
1	15.63	300 $\mu\text{L}$ Calibrator 2	300 $\mu\text{L}$
0	0		600 $\mu\text{L}$

**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 Bovine 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 should be stored at 4°C.

## 4. Enzyme-Antibody Conjugate

Undiluted anti-Haptoglobin-HRP conjugate should be stored at 4°C in the dark and diluted immediately prior to use. The undiluted conjugate solution is stable until the expiration date.

## 5. TMB Substrate Solution

The TMB Substrate Solution should be stored at 4°C in the dark and is stable until the expiration date. Protect from light.

## 6. Stop Solution

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

## 7. Microtiter Plate

Anti-bovine 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. Bovine Haptoglobin Calibrator

**The lyophilized Bovine 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.

## 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.

## 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. For a single step determination a dilution of 1:50 is appropriate for most serum/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 1:50 dilution of sample, transfer 5  $\mu$ L of sample to 245  $\mu$ L of 1X Diluent. This gives you a 1:50 dilution. Mix thoroughly.

### Procedure

**Bring all reagents to RT before use.**

The Calibrators and the test sample(s) should be loaded into the ELISA wells as quickly as possible to avoid a shift in OD readings. Using a multichannel pipette would reduce this occurrence.

1. Pipette 100  $\mu$ L of
  - Calibrator 0 (0.0 ng/mL) in duplicate
  - Calibrator 1 (15.63 ng/mL) in duplicate
  - Calibrator 2 (31.25 ng/mL) in duplicate
  - Calibrator 3 (62.5 ng/mL) in duplicate
  - Calibrator 4 (125 ng/mL) in duplicate
  - Calibrator 5 (250 ng/mL) in duplicate
  - Calibrator 6 (500 ng/mL) in duplicate
  - Calibrator 7 (1,000 ng/mL) in duplicate
2. Pipette 100  $\mu$ L of diluted sample (in duplicate) into pre designated wells.
3. Incubate the Microtiter Plate at 22°C (RT) for fifteen (15  $\pm$  2) minutes. Keep plate covered and level 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 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.
6. Pipette 100  $\mu$ L of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at 22°C (RT) for fifteen (15  $\pm$  2) minutes. Keep the 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  $\mu$ L 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  $\mu$ L of Stop Solution to each well.
11. Determine the absorbance at 450 nm of the contents of each well within 30 minutes. 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. 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.

## 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**

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