Bovine haptoglobin

ELISA kit for the quantitative determination
of Bovine haptoglobin
Sandwich test for blood sera and milk
Diagnostic test for cattle
Monowell

I - INTRODUCTION

Haptoglobin is a glycoprotein that is synthesized by hepatocytes during the acute phase of an inflammatory reaction. This protein has the ability to bind haemoglobin. The plasma level of haptoglobin can increase in the course of certain inflammatory reactions (burns, allergies, injuries, lameness, infections, etc.). The haptoglobin concentration in milk can also increase considerably in the case of mastitis. The upper limit of the haptoglobin concentration is considered to be 100 µg/ml in blood serum and plasma and 0.5 µg/ml in milk.

II – PRINCIPLE OF THE TEST

The test uses 96-well microplates sensitised with monoclonal antibody directed specifically against bovine haptoglobin. This antibody ensures specific capture of the protein in the sample (be it blood serum or milk). The entire plate is sensitised by the antibody. The prediluted test samples are incubated on the microplate (1 hour at 21 ± 3°C). A reference curve is prepared by diluting the standard supplied in the kit. This standard is lyophilised. After the first incubation, the plate is washed and incubated for another hour with the conjugate, which is a specific anti-bovine haptoglobin antibody coupled to peroxidase. After this second incubation the plate is washed once more and the chromogen tetramethylbenzidine (TMB) is added. This chromogen has the advantage of being more sensitive than the other peroxidase chromogens and not being carcinogenic. The enzyme reaction is then stopped by acidification and the wells' optical densities are read by a microplate spectrophotometer at 450 nm. The optical density readings for the unknown samples are plotted on the calibration curve to determine the samples' concentrations in nanograms/ml.

III - COMPOSITION OF THE KIT

- **Microplate**: One 96-well microplate. The entire microplate is sensitised with anti-bovine haptoglobin antibody.
- **Washing solution**: One 100-ml bottle of 20x concentrated washing solution. The solution crystallises spontaneously when cold. If only part of the solution is to be used, bring the bottle to a temperature of 21 ± 3°C until disappearance of all crystals, mix the solution well and withdraw the necessary volume. Dilute the buffer 20-fold with distilled or demineralised water. Store the diluted solution between +2°C and +8°C.

- **Dilution buffer**: One 50-ml bottle of 5x coloured, concentrated buffer. If only part of this solution is to be used, mix it well and withdraw the necessary volume. Dilute the concentrated dilution buffer 5-fold with distilled or demineralised water. Store the diluted solution between +2°C and +8°C. If a deposit forms at the bottom of the container filter the solution on Whatman filter paper.
- **Calibrator**: One 500 µl vial freeze-dried
- **Conjugate**: One 12 ml vial of anti-bovine haptoglobin conjugate. The reagent is ready to use.
- **Single-component TMB solution:** One 12 ml vial of the chromogen TMB (tetramethylbenzidine). This reagent is stored between +2°C and +8°C protected from light. It is ready to use.
- **Stop solution**: One 6 ml bottle of 1 *M* phosphoric acid stop solution.

	BIO K 328/1
Microplate	1
Washing solution	1 X 100 ml (20 X)
Coloured Dilution buffer	1 X 50 ml (5 X)
Conjugate	1 X 12 ml (1 X)
Calibrator	1 X 0,5 ml freeze-dried
Single component TMB	1 X 12 ml (1 X)
Stop solution	1 X 6 ml (1 X)

IV - ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

Distilled water, graduated cylinders, beakers, plastic tubes, tube rack, dispenser tips, reagent reservoir for multichannel pipettes, lid, adhesive for microplates, graduated automatic (mono- and multichannel) pipettes, microplate reader, and microplate washer and shaker (optional)

V - PRECAUTIONS FOR USE

- This test may be used for “in vitro” diagnosis only. It is strictly for veterinary use.
- The reagents must be kept between +2°C and +8°C. The reagents cannot be guaranteed if the shelf-life dates have expired or if they have not been kept under the conditions described in this insert.
- The concentrated wash solution and dilution buffer may be stored at room temperature. Once diluted, these solutions remain stable for six weeks if kept between +2°C and +8°C.
- Unused strips must be stored immediately in the aluminium envelope, taking care to keep the desiccant dry and the envelope’s seal airtight. If these precautions are taken, the strips’ activity can be conserved up to the kit’s shelf-life date.
- Do not use reagents from other kits.
- The quality of the water used to prepare the various solutions is of the utmost importance. Do not use water that may contain oxidants (e.g., sodium hypochlorite) or heavy metal salts, as these substances can react with the chromogen.
- Discard all solutions contaminated with bacteria or fungi.
- The stop solution contains 1 M phosphoric acid. Handle it carefully.
- All materials and disposable equipment that come in contact with the samples must be considered potentially infectious and be disposed of in compliance with the legislation in force in the country.
- To guarantee the reliability of the results, one must follow the protocol to the letter. Special care must be taken in observing the incubation times and temperatures, as well as measuring the volumes and dilutions accurately.

VI – PROCEDURE

1- All components must be brought to 21°C ± 3°C before use.
2- Dilute the washing solution 20-fold with distilled water. Make certain that all the crystals have disappeared before using the dilute solution. Dilute the dilution buffer 5-fold with distilled water. Store the two solutions between +2°C and +8°C when they are not being used.
3- Establish the calibration curve as follows: Add exactly 500 µl of distilled water to the contents of the calibrator vial. When the powder has dissolved completely and the liquid is homogeneous, take up exactly 150µl and add 1350 µl of dilution solution (1/10 dilution of the calibrator). Next establish the calibration curve as indicated in the table below. It is possible to divide the rest of the serum calibrator (350 µl) into precise 150 µl aliquots and freeze them at -20°C for later use. These 150 µl aliquots may be thawed once only.

Calibrator	300 µl	150 µl	150 µl	150 µl	150 µl	150 µl	150 µl
Dilution buffer		150 µl	450 µl	1.05 ml	2.25 ml	4.65 ml	9.45 ml
Dilution	1:1	1:2	1:4	1:8	1:16	1:32	1:64
Concentration	360 ng/ml	180 ng/ml	90 ng/ml	45 ng/ml	22.5 ng/ml	11.25 ng/ml	5.6 ng/ml

The accuracy of the ELISA depends to a great extent on the care taken in making these dilutions. For reliable results, using a precision diluter equipped with Hamilton syringes, for example, is highly recommended.
4- Dilution of the unknown samples: Prepare the dilutions of the unknown samples in the dilution solution. Provide for at least 200 µl per dilution (100 µl per well). The dilution factors to apply will depend on the approximate haptoglobin concentrations in the samples. It is crucial that the optical densities of the test samples are located between the highest and lowest values on the calibration curve. Ideally, the optical density reading of an unknown sample should be close to the calibration curve's turning point. To determine approximately how much to dilute unknown samples, use the values given in the following table:

	Dilution factor
Cow's milk:	1/10
Blood serum:	1/1000

For serum samples from animals for which the clinical examination findings point to the likelihood of a localised inflammation (mastitis, lameness, arthritis, metritis, etc.), it is preferable to use a 3000-fold rather than a 1000-fold dilution factor. Take account of this dilution factor when computing the concentration.
5- Pipette the 7 dilutions of the calibration curve onto the microplate at the rate of 100 µl per well.
6- Do the same for the diluted samples. Cover with a lid and incubate the plate at 21°± 3°C for one hour.
7- Rinse the plate with the washing solution prepared as instructed in the section "Composition of the Kit". To do this, dispose of the microplate's contents by flipping it sharply over a container filled with an inactivating agent. Let the microplate drain upside-down on a sheet of clean absorbent paper so as to eliminate all liquid. Add 300 µl of the washing solution, and then empty the plate once again by flipping it over above the containment vessel. Repeat the entire operation two more times, taking special care to avoid the formation of bubbles in the wells. After these three washes, go on to the next step.
8- Distribute the conjugate over the microplate at the rate of 100 µl per well. Cover with a lid and incubate the plate at 21°± 3°C for one hour.
9- Rinse the plate as described in Step7.
10- Distribute TMB over the microplate at the rate of 100 µl per well. The solution must be perfectly colourless. If a blue tinge is visible, the solution or pipette has been contaminated. Incubate at 21°C ± 3°C without a lid and in the dark for 10 minutes. This time interval is given as a guideline only, for in some circumstances it may be useful to lengthen or shorten the incubation time.
11- Add 50 µl of stop solution to each well. The blue colour will change into a yellow colour.
12- Record the optical densities using a microplate spectrophotometer with a 450 nm filter. The results must be read as quickly as possible after the stop solution has been applied, for in the case of a strong signal the chromogen can crystallise and lead to incorrect readings.

VII – CALCULATING THE RESULTS

In order to calculate the haptoglobin concentrations in the unknown samples, it is preferable to use a computer programme with Log/Logit or 4-parameter curve fitting options. If such a programme is available, key in the haptoglobin concentrations in the calibration curve. Name each sample and indicate its dilution factor. The programme will compute the four parameters of the calibration curve's equation along with its correlation coefficient. Interpolate the values in order to obtain the concentrations of the unknown samples.

Many websites offer free of charge tools to calculate "4 parameter" type curves, such as:
- https://www.myassays.com
- http://elisaanalysis.com
- https://www.mycurvefit.com

VIII – ORDERING INFORMATION

QuantELISA Bovine haptoglobin: 1X 96 tests BIO K 328/1