TECOmedical Group

TECO®
Equine Haptoglobin ELISA

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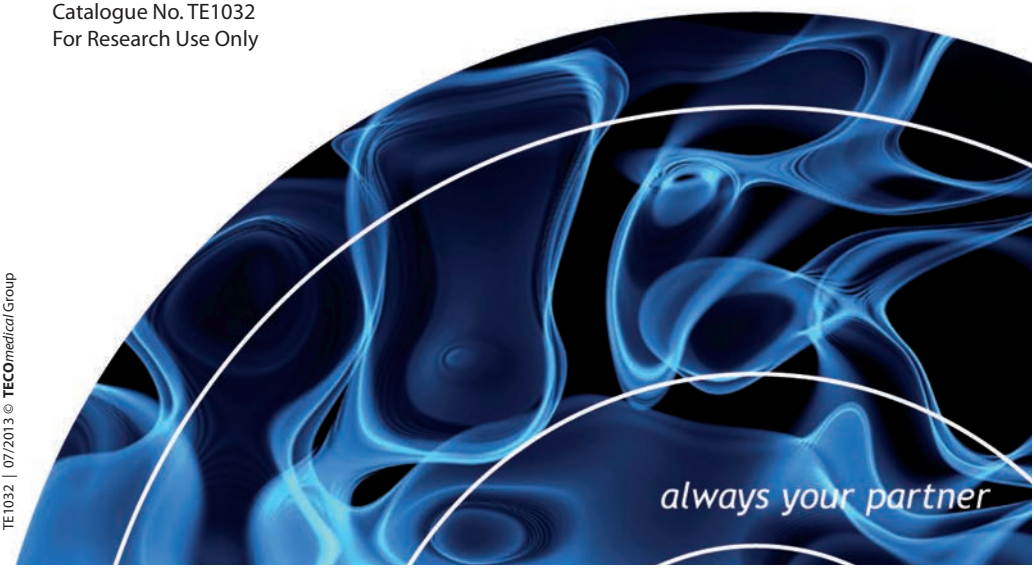
Instructions for use
English

Catalogue No. TE1032
For Research Use Only



Symbol Description

Kit Instructions

Lot Number

Expiry Date

Storage Temperature

Manufacturer

Intended use

TE 1032

Caution: caustic

Tests

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TECO® Equine Haptoglobin ELISA Kit

CONT **Reagents and Materials Supplied:**

Symbol	Description	Format
1	**96-well plate coated with Equine HPT Antibody** 12 break apart strips of 8 wells (12 x 8 in total), in a frame. Ready to use.	**1 plate**
S	**Standard Stock 50x** 50 µg/ml, tinted brown color	**1 x 0.2 ml**
2	**Wash Buffer 50x**	**1 x 30 ml**
3	**Dilution Buffer 10x**	**1 x 30 ml**
4	**Assay Buffer** Ready for use, tinted brown color	**1 x 12 ml**
6	**HRP Antibody Conjugate** Ready for use, tinted brown color	**1 x 12 ml**
7	**TMB Substrate** Ready for use	**1 x 12 ml**
8	**Stop Solution – 1 M HCl** 1 M hydrochloric acid, ready for use	**1 x 12 ml**
	**Kit instruction**	**1 x**

Storage

Store kit at 2–8 °C. Do not freeze. Store unused reagents at 2–8 °C.

Instructions for Use

The TECO® Equine Haptoglobin kit is a sensitive sandwich enzyme linked immunosorbent assay for the quantitative determination of haptoglobin in equine serum.

Background

Haptoglobin, an acute phase protein, is part of immune system-mediated defence mechanisms found in the blood various animal species. Under normal conditions, haptoglobin is either absent from the blood or present at very low levels. However, haptoglobin can increase significantly in response to acute infection, inflammation or trauma. Several functional properties of haptoglobin have been described. The major biological function of haptoglobin is to bind free hemoglobin in an equimolar ratio with very high affinity to prevent hemoglobin-mediated renal parenchymal injury and loss of iron following intravascular hemolysis[1]. The complex of haptoglobin with hemoglobin is metabolized in the hepatic reticuloendothelial system. Biosynthesis of haptoglobin occurs not only in the liver, but also in adipose tissue and lung, providing antioxidant and antimicrobial activity[2].

Different studies showed differences in average measurements of serum haptoglobin according to the horses' age. In foals the reference range of haptoglobin is significant higher compared to adult horses. Comparisons within adult horses showed no differences between the different age-groups[3]. In equine medicine haptoglobin is used as one of the major indexes in addition to fibrinogen and serum amyloid A to examine the acute phase reaction in horses[4]. Pollock et al. used haptoglobin to assess postoperative inflammatory reactions in a clinical trial. They describe a slow increase in serum concentration of haptoglobin starting 24 till 48 hours after surgery. Haptoglobin can be used as a global parameter for inflammatory processes, since it is not disease-specific. This is underlined by several studies examining the haptoglobin serum levels in relation to different diseases like respiratory[5] and orthopedic disorders[4] or surgical interventions[3].

All studies strengthened the statement that haptoglobin is an alternative to serum amyloid A or fibrinogen as a diagnostic instrument for an acute phase reaction. In combination with serum amyloid A haptoglobin can be used to diagnose the inflammatory kinetic. Haptoglobin can as well be used to monitor the response of treatments.
In equine medicine haptoglobin as acute phase protein is helpful in diagnostic and therapy of various diseases.

[1] Lim SK (2001)
Consequences of haemolysis without haptoglobin.
Redox Rep 6: 375-378.

[2] Dobryszycka W (1997)
Biological Functions of Haptoglobin - New Pieces to an Old Puzzle.
Eur J Clin Chem Clin Biochem 35: 647-654.

[3] Pollock PJ, Prendergast M, Schumacher J, Bellenger CR (2005)
Effects of surgery on the acute phase response in clinically normal and diseased horses.
Vet Rec 23;156: 538-542..

[4] Hultén C, Grönlund U, Hirvonen J, Tulamo RM, Suominen MM, Marhaug G, Forsberg M (2002)
Dynamics in serum of inflammatory markers serum amyloid A (SAA), haptoglobin, fibrinogen an α2-globins during induced noninfectious arthritis in the horse.
Equine vet J 34: 699-704.

[5] Lavoie-Lamoureux A, Leclere M, Lemos K, Wagner B, Lavoie JP (2012)
Markers of Systemic Inflammation in Horses with Heaves.
J Vet Intern Med 26: 1419–1426.

Assay Principle

The TECO® Equine haptoglobin EIA Kit is a 96 well sandwich ELISA product. The assay uses affinity purified anti-horse haptoglobin antibodies immobilized at solid phase (microtiter wells). Pre-diluted samples are incubated in the microtiter wells for 60 min. The microtiter wells are subsequently washed, and horseradish peroxidase (HRP) conjugated anti-haptoglobin antibodies are added and incubated for 30 minutes for detection. After incubation, the unbound HRP-labeled antibodies are washed away.

TMB substrate is added which reacts with the HRP and resulting in a concentration-depended color level. The reaction is stopped with HCl changing the blue color to yellow, and the plate is read using a plate reader at 450 nm.

Color development is proportional to the amount of haptoglobin in the sample.

Materials Required and not Supplied

- Pipette capable of accurately dispensing 10-100 µl and 100-1000 µl
- Multichannel pipette for 100 µl
- Graduated cylinders for reconstituting or diluting reagents
- Automatic washer or equivalent plate washing system
- Distilled or deionized water
- Vortex mixer
- ELISA plate shaker (orbital shaker, 500 rpm)
- ELISA plate reader suitable for 96 well formats and capable of measuring at 450 nm (Reference Filter: 590–650 nm).
- ELISA plate reader software for data generation and analysis

Warnings and Precautions

This kit is intended for research use only

Follow the instructions carefully.

Observe expiration dates stated on the labels and the specified stability for reconstituted reagents. Refer to "Materials Safety Data Sheet" for more detailed safety information.

TECOmedical AG is not liable for loss or harm caused by non-observance of the Kit instructions.

1. For Research Use Only. Not for use in diagnostic procedures.
2. Treat all specimen samples as potentially biohazardous material. Follow Universal Precautions when handling contents of this kit and any patient samples
3. Material of animal origin used in the preparation of this kit has been obtained from animals certified as healthy but these materials should be handled as potentially infectious.
4. Disposal of containers and unused contents should be performed in accordance with federal and local regulatory requirements.
5. Use the supplied reagents as an integral unit prior to the expiration date indicated on the package label.
6. Store assay reagents as indicated.
7. Do not use coated strips if pouch is punctured.
8. It is recommended to test each sample in duplicate.
9. Use of multichannel pipettes is recommended to ensure the timely delivery of liquids, however, do NOT use a multichannel pipette for plate washing steps.
10. a) 1 M hydrochloric acid is caustic and can cause severe burns.
    b) Handle TMB (3,3‘,5,5‘-tetramethylbenzidine) with care, and minimize exposure to light. Do not ingest. Avoid contact with skin, eyes, or clothing. If contact is made, wash with water. If ingested, call a physician.
11. As preservative 5-Bromo-5-nitro-1,3-dioxane (0,06 %) is used for the antibody and Sample Diluent. Do not ingest. Avoid contact with skin, eyes, or clothing. If contact is made, wash with water. If ingested, call a physician.

Preparation of Reagents

1 **Microwell Plate Coated with Equine HPT specific Antibody with plate cover**
12 break apart strips of 8 wells (96 in total) in a frame and sealed in a foil bag. Fit strip wells firmly into the frame. After opening, immediately return any unused wells to the original foil package and seal. Store at 2–8 °C until expiration date.

S **Standard Stock 50x**
1 vial containing 0.2 ml equine HPT (50 µg/ml)
Store at 2–8 °C until expiration date.
See Standard preparation.

2 **Wash Buffer 50x**
1 vial of 30 ml buffer, 50 x concentrated. Precipitation may occur in the buffer; resolve before use by warming up and mixing. Bring the vial content to 1500 ml with deionized or distilled water. The diluted washing solution is stable for 4 weeks at 2–8 °C. Store undiluted buffer at 2–8 °C until expiration date.

3 **Dilution Buffer 10x**
1 vial of 30 ml. 10x concentrated. Dilute 1:10 with deionized or distilled water e.g. 30 ml + 270 ml deionized or distilled water. The diluted solution is stable for 4 weeks at 2–8 °C. Store undiluted buffer at 2–8 °C until expiration date.

4 **Assay Buffer**
1 vial of 12 ml. Ready for use. Store at 2–8 °C until expiration date.

6 **HRP Antibody Conjugate**
1 vial of 12 ml. Ready for use. Store at 2–8 °C until expiration date.

7 **TMB Substrate**
1 vial of 12 ml of H_2O_2 and stabilized 3,3‘,5,5‘-tetramethylbenzidine. Ready for use.
Store at 2–8 °C until expiration date.

8 **Stop Solution – 1 M HCl**
1 vial of 12 ml of 1 M hydrochloric acid. Ready for use.
Store at 2–8 °C until expiration date.

Preparation and Stability of Serum Samples

Sample Type and Preparation: Serum, Plasma and Cell Culture
Non-lipemic samples are recommended. Centrifuge collected blood samples within 4 hours.
Predilute samples 1:10 000 with Dilution Buffer 1x 3 in two dilution steps.

Step	Dilution Factor	Dilution Buffer
1	100	990 µl Dilution Buffer + 10 µL Sample
2	10000	990 µl Dilution Buffer + 10 µL Sample (1:100)

Sample Stability
- Maximum 3 days at 2–8 °C or room temperature
- Maximum 12 months at -20 °C
- Longer Storage at -80 °C
- Maximum 3 freeze/thaw cycles

Preparation of Standard (in Dilution Buffer)
Standards have to be prepared freshly before use.
Use the Dilution Buffer 1x 3 delivered by TECOmedical for Standard preparation.

The Standard vial S contains 50 µg/ml equine HPT.
Preparation of the standard curve **with Dilution Buffer 1x** 3

ID	Concentration	Dilution Buffer
Standard A	1000 ng/mL	980 µl Dilution Buffer + 20 µL Standard Stock
Std B	400 ng/mL	300 µL Dilution Buffer + 200 µL Std A
Std C	160 ng/mL	300 µL Dilution Buffer + 200 µL Std B
Std D	64 ng/mL	300 µL Dilution Buffer + 200 µL Std C
Std E	26 ng/mL	300 µL Dilution Buffer + 200 µL Std D
Std F	0 ng/mL	Dilution Buffer

Assay Procedure

It is recommended that all determinations (Standards and samples) are assayed in duplicate. When performing the assay, Standards and samples should be pipetted as fast as possible (< 15 minutes).
To avoid distortions due to differences in incubation times, Substrate Solution and Stop Solution should be added to the plate in the same order and with the same time interval. Before use, allow all reagents to stand at room temperature (20–25 °C) for at least 30 minutes. During all incubation steps, plates should be sealed with the adhesive foil or a plastic cover. For light protection, incubate in a dark chamber or cover plate with aluminium foil.

1. Allocate the wells of the Microwell Plate for Standards and samples

2. Pre-wash the microassay strips as follows:
   a. Using a wash bottle or automated plate washing device, add approximately 350 µL Wash Buffer to each well.
   b. Incubate the wells for two minutes at 20-25°C.
   c. Aspirate the contents from each well.
   Invert the plate and tap firmly on absorbent paper to remove any remaining liquid.

3. Add **100 µl** Assay Buffer to each well (multichannel pipette)

4. Pipette **20 µl** of each Standard (A till F) and diluted samples into the corresponding wells.

5. Incubate the plate for **1 h** at room temperature (20–25 °C) on a shaker (500 rpm).

6. After incubation, aspirate the wells by using a plate washer or manually decant by inverting the plate. Wash the wells 3 times with 350 µl diluted Wash Buffer per well. After the last wash cycle tap the inverted wells on a dry absorbent surface to remove excess wash solution. The use of an automatic plate washer is recommended.

7. Add **100 µl** of HRP Antibody Conjugate (multichannel pipette).

8. Incubate the plate for **30 min** in the dark at room temperature (20–25 °C) on a shaker (500 rpm).

9. After incubation, aspirate the wells by using a plate washer or manually decant by inverting the plate. Wash the wells 3 times with 350 µl diluted Wash Buffer per well. After the last wash cycle tap the inverted wells on a dry absorbent surface to remove excess wash solution. The use of an automatic plate washer is recommended.

10. Pipette 100 µl of the TMB Substrate Solution in each well (multichannel pipette).

11. Incubate the plate for **15–30 min** in the dark at room temperature (20–25 °C) on a shaker (500 rpm).

12. Stop the reaction by adding **100 µl** of Stop Solution (multichannel pipette).

13. Measure the color reaction within **10** min at 450 nm (reference filter between 590–650 nm).

Result Analysis

Equine Haptoglobin Concentration

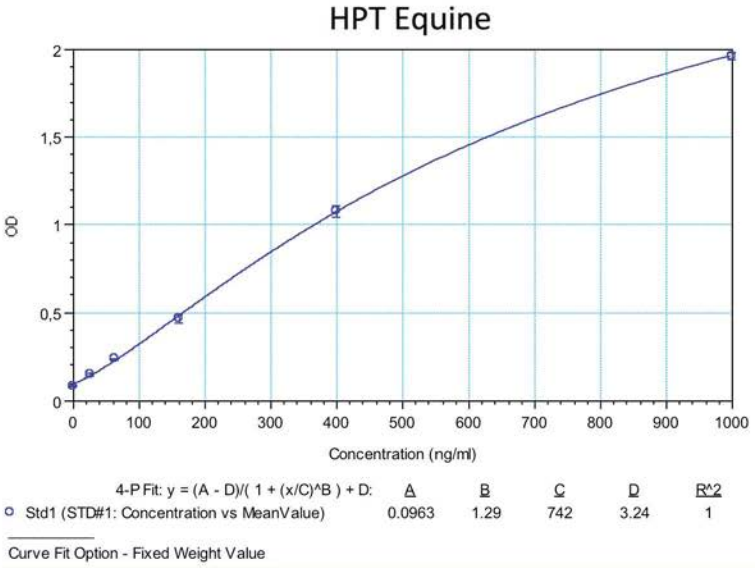
A Standard curve can be established by plotting standard concentration on the x-axis (linear scale) against the absorbance of the Standards on the y-axis (linear scale). A 4-parameter curve fit should be used for automatic data reduction. The equine HPT concentration of samples will be obtained by multiplying the value read off the standard curve by the dilution factor used for the given sample.

Samples with higher absorbance values than Standard A should be tested again with a higher dilution.

Typical Standard Curve

(Example only, not for use in calculation of actual results)

Standards	Concentration (ng/ml)	Absorption at 450 nm
Standard A	1000 ng/mL	1.963
Std B	400 ng/mL	1.077
Std C	160 ng/mL	0.466
Std D	64 ng/mL	0.233
Std E	26 ng/mL	0.147
Std F	0 ng/mL	0.085



Observed Values

Serum samples from 22 healthy horses were tested.
The mean HPT concentrations were 0.62 ± 0.13 mg/ml.
Based on these values a cut-off of 1 mg/ml (mean + 3 SD) has been defined.

Note: Data Normal Range | Steinig et al., University of Veterinary Medicine Hannover - Foundation, Germany

Test Performance

Precision
(Inter assay)

Sample (serum)	Mean (ng/ml)	SD (ng/ml)	CV (%)
Sample 1	91	6.9	7.5
Sample 2	99	7.5	7.6
Sample 3	115	8.0	7.0
Sample 4	120	7.9	6.6

(Intra assay)

Sample (serum)	Mean (ng/ml)	SD (ng/ml)	CV (%)
Sample 1	91	4.0	4.3
Sample 2	99	3.4	3.5
Sample 3	115	4.1	3.6
Sample 4	120	3.4	2.8

Interferences
No interference with hemoglobin in hemolytic samples.

Detection Limit
The kit detection limit was calculated in 9 Runs.
The mean detection limit is defined as Standard F (0 ng/ml) plus 3 SD: 11.3 ng/ml.

Spike Recovery
The mean recovery of equine HPT spiked to normal samples 106 %.

Sample	Concentration ng/ml	Added ng/ml	Expected ng/ml	Measured ng/ml	Spike Recovery %
Sample 1	89	433	522	572	110
Sample 2	94	433	528	558	106
Sample 3	107	433	541	583	108
Sample 4	113	433	547	570	104
Sample 5	104	433	538	576	107
Sample 6	115	433	548	558	102

Dilution Recovery
The mean dilution recovery of equine samples was 98 %.

Sample	Dilution	Measured ng/ml	Expected ng/ml	Recovery %
Sample 1	2500 5000 10000	329 159 89	 164 82	 97 108
Sample 2	2500 5000 10000	356 173 94	 178 89	 97 106
Sample 3	2500 5000 10000	423 203 107	 212 106	 96 101
Sample 4	2500 5000 10000	453 214 113	 226 113	 94 100
Sample 5	2500 5000 10000	438 199 104	 219 109	 91 95
Sample 6	2500 5000 10000	468 218 115	 234 117	 93 98

TECO® Equine Haptoglobin ELISA

Assay Procedure – Quick Guide

- Bring samples and reagents to room temperature. Mix the samples well.
- Dilution Buffer: Dilute 1:10 with deionized or destilled water.
- Dilute equine HPT Standard **S** according to instruction with Dilution Buffer 1x.
- Washing Buffer: Dilute 1:50 with deionized or destilled water.
- Predilute samples with Dilution Buffer (e.g. 1:10 000).

Prepare the required number of Assay Strips.

Immediately before use pre-wash the microassay strips once with **350 µl** with **2 min.** soake time, aspirate and tap the inverted wells on a clean dry absorbent surface.

Pipette **100 µl** diluted Assay Buffer into each well.
Add **20 µl** standards and diluted samples into assay wells.

Incubate **1 h** at 20-25 °C on a shaker (500 rpm).

Aspirate and wash **3 x** with **350 µl** Wash Buffer, aspirate and tap the inverted wells on a clean dry absorbent surface.

Pipette **100 µl** HRP Antibody Conjugate into each well.

Incubate **30 min** at 20–25 °C in the dark on a shaker (500 rpm)

Aspirate and wash **5 x** with **350 µl** Wash Buffer, aspirate and tap the inverted wells on a clean dry absorbent surface.

Pipette **100 µl** TMB Substrate Solution.

Incubate **15-30 min** at 20-25°C in the dark on a shaker (500 rpm).

Pipette **100 µl** Stop Solution.

Read the optical density at **450 nm**, using a referencefilter between 590-650 nm.
Analyze theassay results using a 4- parameter curve fit: y= (A-D)/(1+(x/C)B)+D
Calculations: measured value x dilution

Please read Kit instruction before using the Quick Guide