"PHASE" ™ Haptoglobin Assay Cat. No. TP-801

Intended Use

This is a colorimetric assay designed to quantitatively measure the concentration of the acute phase protein haptoglobin in serum and plasma from a range of animal species.

The product is for *in vitro* research purposes only.

Application

The activation of the body's immune system-mediated defence mechanisms is termed the acute phase response. Haptoglobin is one of a series of acute phase proteins that is found in the blood of a range of animal species. Under normal conditions, it is either absent from the blood or present at very low levels. Depending on the species normal ranges can vary from less than 0.05 mg/ml in cattle to 0.3 – 3.5mg/ml in dogs. However, haptoglobin can increase significantly in response to acute infection, inflammation or trauma. The rise in serum or plasma haptoglobin and the continuous monitoring of this during the acute phase response gives valuable information to the clinician or researcher.

Test Principle

Free haemoglobin exhibits peroxidase activity, which is inhibited at a low pH. Haptoglobin present in the specimen combines with haemoglobin and at a low pH preserves the peroxidase activity of the bound haemoglobin. Preservation of the peroxidase activity of haemoglobin is directly proportional to the amount of haptoglobin present in the specimen. This assay can be performed in manual or automated formats.

Reagent Materials Provided

1. Reagent 1 (R1) 1 x 14ml stabilised Haemoglobin. (Ready to use)
2. Reagent 2 (R2) 1 x 20ml Chromogen reagent (Ready to use)
3. Calibrator 1 x 0.5ml Haptoglobin Calibrator (2.5mg/ml).
4. Sample/Calibrator Diluent 1 x 12ml Phosphate Buffered Saline Diluent (Ready to use).

Additional Materials Required

1. Serum or plasma collection equipment.
2. Automated analyser (A_{600nm}) or Microplate reader (A_{630nm}).
3. Accurate micropipettes or multichannel pipettes and disposable tips to deliver 0-10µl and 50-200µl
4. Test tubes.
5. Timer.
6. Graph paper: Linear (Cartesian)
7. 96 well microtitre plate or strips.
8. Vortex
9. Centrifuge

Warnings and Precautions

- This test is for *in vitro* research use only.
- Avoid contact with eyes, skin and clothing. Wash hands thoroughly after handling and when finished. Avoid ingestion of reagents. Never pipette by mouth and wear disposable latex gloves and eye protection where appropriate.
- Reagent 2 contains reagents that may irritate the skin or mucous membranes. Any reagent which comes in contact with skin should be washed off with water immediately.
- Some reagents contain thimerosal and may be toxic if ingested.

Procedural

- Do not use kit or individual reagents past their expiry date.
- Do not mix or substitute reagents from different kit lot numbers.
- Deviation from protocol provided may cause erroneous results.
- Samples should be stored refrigerated, or frozen if they are not to be analysed shortly after collection. Avoid repeated freeze thaw cycles.
- When possible avoid the use of haemolysed or lipaemic sera. If large amounts of particulate matter are present this should be removed by centrifugation prior to assay.
- It is recommended that all calibrators and samples are run in duplicate.
- Allow all reagents to come to room temperature **(20 – 25^{0}C)** and mix well before use.
- Avoid leaving reagents in direct sunlight and/or above 4^{0}C for extended periods.
- Cover or cap all reagents when not in use.
- Always use clean, preferably disposable, glassware for all reagent preparation.
- Care must be taken not to contaminate components and always use fresh tips for each sample and component.
- In manual format ensure that the bottom surface of the well is clean and dry before reading. Ensure no bubbles are present in the wells prior to reading.
- Before commencing the manual assay format an identification and distribution plan should be established. It also recommended labelling each strip to enable identification.
- Read absorbances immediately after completion of the assay. Do not allow the calibration curve to over develop and undergo a wavelength shift (change from blue to light brown colour).

Reagent Preparation & Use

Haemoglobin (R1)
This reagent is provided in a ready to use format (***Reagent 1***).

Chromogen (R2)
This reagent is provided in a ready to use format (***Reagent 2***).

Calibrator/sample diluent
This reagent is provided in a ready to use format.

Calibrator (Microplate Method)

Calibration is run in the form of a standard curve for each assay. The standard curve dilution series is prepared manually or automatically on an automated analyser.

For the manual method label five tubes with numbers C1-C5, corresponding to haptoglobin standards 2.5, 1.25, 0.625, 0.312 and 0 mg/ml respectively. Calibrators for this test format are prepared by following the steps provided in the table:

Tube No:	Volume Calibrator	Volume Diluent	Tube Concentration
C1	50µl of stock	-	2.5 mg/ml
C2	50µl of stock	50µl	1.25mg/ml
C3	50µl C2	50µl	0.625mg/ml
C4	50µl C3	50µl	0.312mg/ml
C5	-	50µl	0mg/ml

Calibrator (Automated Methods)

- For automated procedures single point calibration may be used by dilution of the 2.5mg/ml standard 1:1 to give a 1.25mg/ml calibrator.
- Alternatively, use only 0, 0.625 and 2.5mg/ml calibrator. (Haptoglobin standard of 0.625mg/ml can be prepared by adding 50ul of 2.5mg/ml standard to 150ul of calibrator diluent).

- Multi-point calibration curves can also be used.
- Note: Account should be taken of the fill volume of calibrator material which may be required depending on the automated system in use.

Assay the calibrators in the context of an internal calibration programme.

Sample Preparation

- This method has demonstrated serum/plasma equivalence using lithium-heparin blood collection tubes for plasma.
- Blood samples may be kept for up to 24 hours before separation of serum.
- It is recommended that serum is removed from the clot or cells as soon as possible after collection. Serum may be stored at 2-8^{o}C for up to 24 hours before screening or alternatively stored long-term frozen at -20^{o}C for up to one year.
- For most species, samples are tested neat and only require dilution if the haptoglobin level is above the highest calibrator concentration. However for canine, use of a starting sample dilution of 1:5 is recommended by adding 10µl of sample to 40µl of calibrator/sample diluent.
- It is important that all samples are brought to room temperature and vortexed vigorously to ensure accurate determination of the haptoglobin concentration.
- Do not use grossly haemolysed or lipaemic samples.

Test Procedure

A. Manual method (microplate or spectrophotometric)

Note: Addition of Reagent 1 and Reagent 2 should be made with a multi-channel or repeating pipette.

1. Transfer 7.5ul of each prepared calibrator (0-2.5mg/ml) along with test specimens, in duplicate, to the blank microplate.
2. Add 100ul of Reagent 1 to each microwell. Tap the microplate gently to ensure mixing of calibrators/specimens and haemoglobin.
3. Add 140ul Reagent 2 to each microwell. Incubate for 5 minutes at room temperature (20-25^{o}C).
4. Read immediately at 600-630nm.

B. Automated Method (2 reagent procedure).

1. Dispense the required amount of Reagent 1 and Reagent 2 into the appropriate storage vessels on the instrument.
2. Aliquot the required volume of sample, controls and calibrator into the appropriate sample cups.
3. Read at 600-630nm while the calibration curve is developing, preferably at the peak of the calibration curve. **Caution:** Do not let the calibration curve 'over develop' and undergo a wavelength shift (change in colour from blue to light brown)

Test Temperature

The test can be performed at 25^{o}C, 30^{o}C or 37^{o}C on analyzers or at room temperature on the manual format.

Interpretation of Results

- Calculate the mean absorbance for each sample, control or standard.
- Generate a calibration curve by plotting absorbance (600-630nm) versus haptoglobin concentration (mg/ml) to facilitate calculation of haptoglobin concentration in test specimens.
- Draw the best smooth curve through these points to construct the standard curve.
- Determine the concentrations of the test samples and controls from the standard curve by reading specimen values directly from the calibrator curve and then multiplying the interpolated value by the appropriate dilution factor.

- Samples that have a signal greater than the highest standard, or fall on the non-linear part of the curve, should be further diluted in diluent buffer and re-analyzed.

Expected Values

SPECIES	NORMAL RANGE (mg/ml)	ACUTE RANGE (mg/ml)
Bovine	0.00-0.05	0.10-3.00
Canine	0.30-3.50	4.00-9.00
Rodent	0.25-0.51	0.80-1.80
Murine	0.00-0.10	0.30-2.00
Feline	0.70-2.00.	3.00-10.0
Porcine	0.00-2.20	3.00-8.00

- Normal ranges for a variety of species are provided above and were determined on a COBAS MIRA at 37°C. However, each laboratory should establish its own normal range for each species being tested which may vary depending on the species selected for study (e.g. Sprague Dawley V's Wistar rodents).

Waste Management

Please refer to local legal requirements

Quality Control

Good laboratory practice suggests the use of control specimens to ensureproper assay performance.

Laboratories should assess the Haptoglobin Kit using haptoglobin control material and haptoglobin calibrator. A separate set of additional **Haptoglobin** Quality Control sera and Calibrators are available from Tridelta specifically for use with this kit and may be purchased as separate items (Catalogue no. TP801CON and TP801 Cal respectively).

Laboratories should establish their own control range. A provisional range is provided with each set of controls and may be used a reference. However, each laboratory should determine the performance of their measurement system and set an appropriate mean and QC limits for the control materials based on their own data.

The required level of performance is achieved when the analyte values obtained for each control are within the acceptable control range as determined by each laboratory. If results fall outside of the established ranges, assay results are invalid. It is recommended the controls be included in every assay. New lots of control material should be analysed in parallel with the control material in current use. Controls should be monitored and charted on a day-to-day basis to analyse values and trends.

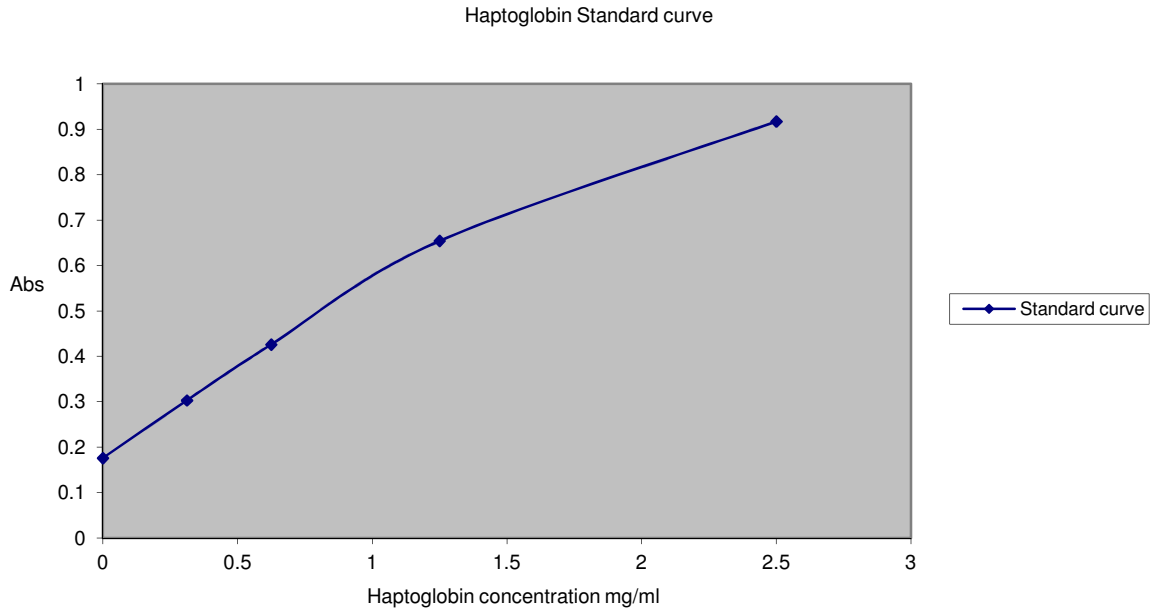
Storage and Stability

The storage and stabilities for the Haptoglobin reagents and calibrators are as follows:

1. **Unopened and stored at 2^0C-8^0C:** Unopened Haptoglobin reagents are stable until the last day of the month of the expiration date printed on the product label.
2. **Opened and stored at 2^0C-8^0C:** Opened Haptoglobin reagents are stable until the last day of the month of the expiration date printed on the product label, if kept closed in their original containers, free from contamination and at the correct temperature.
3. **On-board stability:** Opened Haptoglobin reagents (excluding prepared calibrators) have on-board stability at room temperature (20-25°C) for 1 week .
4. **Calibrators** prepared are stable for 8 hours when stored at room temperature (20-25°C) in closed containers.

5. Protect all reagents from extreme heat or freezing. In order to ensure maximum stability of the Haptoglobin reagents on each automated chemistry analyser, it is important to use proper boats and anti-evaporation tray covers.

Performance Characteristics



Note: An example of a typical standard curve is presented above.
This should not be used in determination of haptoglobin.

Intra assay Variation

(n=32)	Mean Hp Concentration (mg/ml)	Standard Deviation	Coefficient of Variation (%)
Low	0.59	0.02	5.3
High	1.30	0.10	6.3

Inter assay Variation

(n=64)	Mean Hp Concentration (mg/ml)	Standard Deviation	Coefficient of Variation (%)
Low	0.59	0.02	5.7
High	1.26	0.10	4.1

Analytical Sensitivity

Analytical sensitivity has been determined as 0.005 mg/ml Haptoglobin. This value was determined by the addition of two standard deviations of the mean OD obtained when the zero calibrator was assayed 32 times.

Limitation of Test

• Serum or plasma samples are recommended for use in this test. However, to eliminate potential discrepancies it is recommended that any study which starts with a particular matrix i.e. serum or plasma, should continue to use the same matrix for the duration of the investigation.
• Mildly hemolysed samples may be used in the test. However, grossly hemolysed samples (haemoglobin >2.5 g/l) should be avoided as results may not be reliable

Other "PHASE" acute phase assays available from Tridelta:

TP-801-Cal	Haptoglobin	Haptoglobin calibrator (2.5mg/ml) for Haptoglobin kit (TP-801)
TP-801-Con	Haptoglobin	Haptoglobin controls for Haptoglobin kit (TP-801)
TP-803	CRP-Canine	C-Reactive Protein EIA kit specific for canine.
TP-803-Con	CRP-Canine	Canine CRP controls for Canine CRP EIA kit (TP-803).
TA-901	CRP-Porcine	C-Reactive Protein EIA kit specific for porcine.
TP-802M	Murine SAA	Serum Amyloid A EIA specific for mouse.
TP-802	SAA	Multispecies Serum Amyloid A immunoassay.
TP-802-Con	SAA	SAA controls for Multispecies SAA kit (TP802)
TP-807	Milk MAA	Amyloid A EIA specific for bovine milk.

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