

# Bovine Haptoglobin (Hpt) ELISA

**Cat No: K04-0121**

ver1.0

**Principle:**

The method employs sandwich ELISA technique. Monoclonal antibodies are pre-coated onto microwells. Samples and standards are pipetted into microwells and Bovine Haptoglobin (Hpt) present in the sample are bound by the antibodies. Biotin labeled antibody is added and followed by Streptavidin:HRP is pipetted and incubated to form a complex. After washing microwells in order to remove any non-specific binding, the substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Bovine Haptoglobin (Hpt) in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

**Intended Use:**

This Kit is used to assay the level of Bovine Haptoglobin (Hpt) in Bovine serum and plasma and other biological samples. The Kit is For Laboratory / Research Use Only.

**Materials provided in the Kit:**

1. Anti-Bovine HPT Coated Microtiter Plate (96 wells) - 1 no
2. Bovine HPT Standard (lyophilized, concentrated, 4000 ng/ml) - 2 vials
3. Biotinylated Anti-HPT (concentrated) - 120 ul
4. Streptavidin:HRP Conjugate (concentrated) - 120 ul
5. Standard Diluent - 20 ml
6. Biotin Antibody Dilution Buffer - 12 ml
7. HRP Conjugate Dilution Buffer - 12 ml
8. (20X) Wash Buffer - 25 ml
9. TMB Substrate - 12 ml
10. Stop Solution - 12 ml
11. Instruction Manual

**Materials to be provided by the End-User:**

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul
3. Deionized (DI) water
4. Wash bottle or automated microplate washer
5. Clean tubes and Eppendorf tubes
6. Precision single and multi-channel pipette and disposable tips.
7. 37°C incubator
8. Timer.

**Storage Information:**

1. For unopened kit: All the reagents should be kept according to the labels on vials. The Standard, Biotinylated Antibody, HRP Conjugate and 96-well plate should be stored at -20°C upon receipt while the others should be at 2-8°C.
2. For opened kits: Once the kit is opened, the remaining reagents still need to be stored according to the above storage conditions. In addition, return the unused wells to the foil pouch containing the desiccant pack and reseal along entire edge of zip-seal.
3. All the reagents and wash solutions are stable until the expiration date of the kit.
4. 20 minutes prior before use, bring all components to room temperature (18-25°C). Store all the components of the kit at its appropriate storage condition after use.
5. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

**Health Hazard Warnings:**

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details.
2. To reduce the likelihood of blood-borne transmission of infectious agents, handle all samples in accordance with NCCLS regulations.

**Specimen Collection and Storage:**

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

1. **Serum-** Place whole blood sample at room temperature for 2 hours or put it at 2-8°C overnight and centrifugation for 20 minutes at approximately 1000xg, Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.
2. **Plasma-** Collect plasma using EDTA-Na<sub>2</sub> or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000xg at 2-8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.
3. **Tissue Homogenates-** As hemolysis blood has relation to the assay results, it is necessary to remove residual blood by washing tissue with pre-coating PBS buffer (0.01M, pH=7.4). Mince tissue after weighing it and get it homogenized in PBS (the volume depends on the weight of the tissue Normal 9 ml PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitors are recommended to add into the PBS) with a glass homogenizer or ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disruptor or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 minutes at 5000xg to get the supernatant. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3 mg,
4. **Cell Culture Supernatant-**Centrifuge supernatant for 20 minutes at 1000xg at 2-8°C to remove insoluble impurity and cell debris. Collect the clear supernatant and carry out the assay immediately.
5. **Cell Culture Lysate-** Commercial RIPA kits are recommended to follow the instructions provided. Generally 0.5 ml RIPA lysis buffer would be appropriate to 2x10<sup>6</sup> cells, DNA must be removed. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3 mg.
6. **Other Biological Fluids-** Centrifuge samples for 20 minutes are 1000xg at 2-8°C. Collect supernatant and carry out the assay immediately.

**Note:** Samples to be used within 5 days can be stored at 2-8°C, besides that, samples must be stored at -20°C (assay ≤1 month) or -80°C(assay≤2 months) to avoid loss of bioactivity and contamination. Avoid multiple freeze-thaw cycles. The hemolytic samples are not suitable for this assay.

**Reagent Preparation (all reagents should be diluted immediately prior to use):**

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room temperature before use.
3. To make **Wash Buffer (1X) 500**; dilute 25 ml of **(20X) Wash Buffer in 475 ml of DI water**.
4. **Streptavidin:HRP Conjugate & Biotinylated HPT Antibody** - Briefly spin or centrifuge the Streptavidin:HRP Conjugate & Biotinylated HPT Antibody before use. Dilute them to the working concentration 100-fold with HRP Conjugate Dilution Buffer & Biotin Antibody Dilution Buffer, respectively.
5. **Standards Preparation:** Reconstitute original HPT Standard with 1.0 ml of Standard Diluent. Keep the standard for 10 mins with gentle agitation before making further dilutions. Prepare the additional Standards by serially diluting the standard stock solution as per the below table.

Standard Concentration	Standard Vial	Dilution Particulars
4000 ng/ml	Original Standard	Reconstitute with 1.0 ml Standard Diluent
1000 ng/ml	Standard No.8	250 ul Standard Provided (4000 ng/ml) + 750 ul Standard Diluent
500 ng/ml	Standard No.7	500 ul Standard No.8 + 500 ul Standard Diluent
250 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent
125 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent
62.5 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent
31.2 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent
15.6 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent
0 ng/ml	Standard No.1	500 ul Standard Diluent only

**Procedural Notes:**

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Bovine Haptoglobin (Hpt). High Dose Hook Effect is due to excess of antibody for very high concentrations of Bovine Haptoglobin (Hpt) present in the sample.
3. Avoid assay of Samples containing Sodium Azide ( $\text{NaN}_3$ ), as it could destroy the HRP activity resulting in under-estimation of the amount of Bovine Haptoglobin (Hpt).
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to poor sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

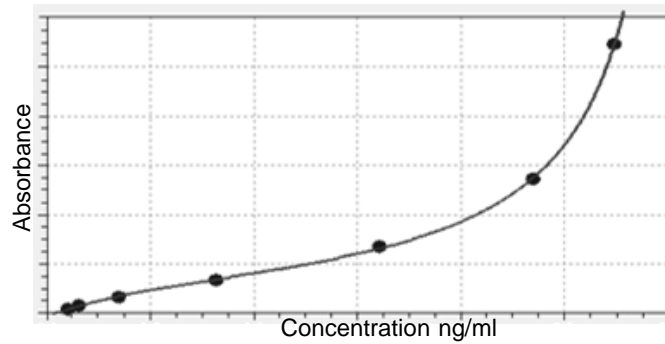
**Assay Procedure:**

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay.
2. Add **100 ul prepared Standards and Samples** to respective wells.
3. Cover the plate with a sealer and incubate for 60 minutes at 37°C.
4. Aspirate the plate. DO NOT WASH.
5. Pipette **100 ul Biotinylated HPT Antibody Working Solution** to all wells.
6. Cover the plate with a sealer and incubate for 60 minutes at 37°C.
7. Aspirate and wash plate 4 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.
8. Pipette **100 ul Streptavidin:HRP Conjugate Working Solution** to all wells. Mix well.
9. Cover the plate with a sealer and incubate for 30 minutes at 37°C.
10. Aspirate and wash as per Step (7) above.
11. Pipette **100 ul TMB Substrate** in all the wells.
12. Incubate the plate at **37°C for 10 minutes**. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.
13. Pipette **100 ul of Stop Solution** to all wells. The wells should turn from blue to yellow in color.
14. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

**Calculation of Results:**

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Use the Net Absorbance (Absorbance of Standard/Sample - Absorbance of Blank) to calculate the Mean Absorbances. Using standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the concentration. If samples were diluted, multiply by the appropriate dilution factor.

Software which is able to generate a cubic spline curve-fit, 4PL or a polynomial regression to the 2<sup>nd</sup> order is best recommended for automated results.

**Typical Graph**

**Precautions:**

Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

**Performance Characteristics:**

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

**Sensitivity:**
**Limit Of Quantification:**

It is defined as the lowest detectable concentration that can be determined with an acceptable repeatability and the LOQ was found to be **5.9 ng/ml**.

**Specificity:**

This assay has high sensitivity and excellent specificity for detection of HPT. No significant cross-reactivity or interference between HPT and analogues was observed.

**Recovery**

Matrices listed below were spiked with certain level of HPT and the recovery rates were calculated by comparing the measured value to the expected amount of HPT in samples.

Matrix	Recovery Range (%)	Average (%)
Serum(n=5)	80-96	87
EDTA Plasma(n=5)	92-104	99
Heparin Plasma(n=5)	86-95	90

**Assay Range:**

**15.6 ng/ml – 1000 ng/ml**

**Precision:**

Intra-Assay: CV<10%

Inter-Assay: CV<12%

**Linearity:**

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of HPT and their serial dilutions. The results were demonstrated by percentage of calculated concentration to the expectation.

Sample	1:2	1:4	1:8	1:16
serum (n=5)	81-94%	79-103%	92-105%	83-97%
EDTA plasma (n=5)	94-105%	96-106%	78-89%	87-102%
heparin plasma (n=5)	80-91%	86-99%	85-101%	84-98%

**LIMITED WARRANTY**

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### ASSAY PROCEDURE

1	Bring all reagents to room temperature before use.		
2	Pipette Standards 1 - 8 Samples	100 ul	100 ul
3	Incubate 60 minutes (37°C)		
4	Pipette Biotinylated Anti-HPT	100ul	100ul
5	Incubate 60 minutes (37°C)		
6	Wash 1X Wash Buffer Decant, 4 x 300 ul		
7	Pipette Streptavidin: HRP Conjugate	100 ul	100 ul
9	Incubate 30 minutes (37°C)		
10	Wash 1X Wash Buffer Decant, 4 x 300 ul		
11	Pipette TMB Substrate	100 ul	
12	Incubate in the dark 10 minutes (37°C)		
13	Pipette Stop Solution	100 ul	
14	Measure 450 within 15 mins		