# Bovine Haptoglobin Immunoassay

**Catalog Number: SEKB-0078**

For the quantitative determination of bovine Haptoglobin concentrations in cell culture supernates, serum, and plasma.

For research use only. Not for use in diagnostic procedures.

**MANUFACTURED AND DISTRIBUTED BY:**

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# TABLE OF CONTENTS

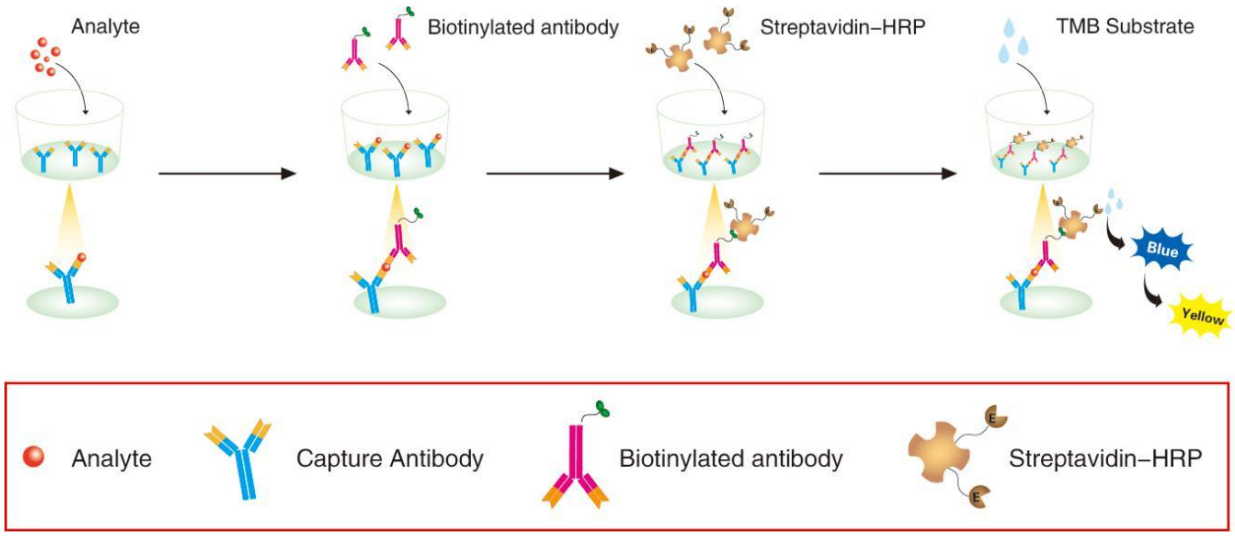
# BACKGROUND

Haptoglobin (Hp) is the protein that in humans is encoded by the *HP* gene. In blood plasma, haptoglobin binds free hemoglobin (Hb) released from erythrocytes with high affinity and thereby inhibits its oxidative activity. The haptoglobin-hemoglobin complex will then be removed by the reticuloendothelial system. In clinical settings, the haptoglobulin assay is used to screen for and monitor intravascular hemolytic anemia. In intravascular hemolysis, free hemoglobin will be released into circulation and hence haptoglobin will bind the hemoglobin. This causes a decline in haptoglobin levels. Conversely, in extravascular hemolysis the reticuloendothelial system, especially splenic monocytes, phagocytose the erythrocytes and hemoglobin is not released into circulation; serum haptoglobin levels are therefore normal. Haptoglobin level is used to determine whether hematology needs to be consulted for hemolytic anemia. This gene encodes a preproprotein that is processed to yield both alpha and beta chains, which subsequently combine as atetramer to produce haptoglobin. Haptoglobin functions to bind free plasma hemoglobin, which allows degradative enzymes to gain access to the hemoglobin while at the same time preventing loss of iron through the kidneys and protecting the kidneys from damage by hemoglobin. For this reason it is often referred to as the suicide protein. Haptoglobin is produced mostly by hepatocytes but also by other tissues. In addition, the haptoglobin gene is expressed in murine, human and bovine adipose tissue.Haptoglobin consists of two α- and two β-chains, connected by disulfide bridges and exists in two allelic forms in the human population, *Hp1* and *Hp2*, the latter one having arisen due to the partial duplication of *Hp1* gene. Hp has been shown to bind hemoglobin with different affinities, with Hp2-2 being the weakest binder. As haptoglobin is indeed an acute-phase protein, any inflammatory process may increase the levels of plasma haptoglobin

# PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Haptoglobin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Haptoglobin present is captured by the coated antibody after incubation. Following extensive washing, a biotin-conjugate antibody specific for Haptoglobin is added to detect the captured Haptoglobin protein in sample. For signal development, horseradish peroxidase (HRP)-conjugated Streptavidin is added, followed by tetramethyl-benzidine (TMB) reagent. Following a wash to remove any unbound combination, and enzyme conjugate is added to the wells. Solution containing sulfuric acid is used to stop color development and the color intensity which is proportional to the quantity of bound protein is measurable at 450nm.

**Schematic diagram：**




# TECHNICAL HINTS AND LIMITATIONS

1. This Solarbio ELISA should not be used beyond the expiration data on the kit label.
2. To avoid cross-contamination, use a fresh reagent reservoir and pipette tips for each step.
3. To ensure accurate results, some details, such as technique, plasticware and water sources should be emphasized.
4. A thorough and consistent wash technique is essential for proper assay performance.
5. A standard curve should be generated for each set of samples assayed.
6. It is recommended that all standards and samples be assayed in duplicate.
7. Avoid microbial contamination of reagents and buffers. Buffers containing protein should be made under aseptic conditions and be prepared fresh daily.
8. In order to ensure the accuracy of the results, the standard curve should be made every time.

# PRECAUTIONS

The Stop Solution suggested for use with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

# KIT COMPONENTS& STORAGE CONDITIONS

| PART | SIZE | STORAGE OF OPENED/ RECONSTITUTED MATERIAL |
|---|---|---|
| **Microwell Plate - antibody coated 96-well Microplate (8 wells ×12 strips)** | 1 plate | Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2 - 8°C** |
| **Standard - lyophilized, 200 ng/ml upon reconstitution** | 2 vials | Aliquot and Store at 2-8°C** for six months |
| **lyophilized Biotin-Conjugated antibody** | 1 vials | Store at 2-8°C **for six months |
| **Concentrated Streptavidin-HRP** | 1 vial | Store at 2-8°C** for six months |
| **Standard /sample Diluent** | 1 bottle | Store at 2-8°C** for six months |
| **Biotin-Conjugate antibody Diluent** | 1 bottle | Store at 2-8°C** for six months |
| **Streptavidin-HRP Diluent** | 1 bottle | Store at 2-8°C** for six months |
| **20 x Wash Buffer Concentrate** | 1 bottle | Store at 2-8°C** for six months |
| **Substrate Solution** | 1 bottle | Store at 2-8°C** for six months |
| **Stop Solution** | 1 bottle | Store at 2-8°C** for six months |
| **Plate Cover Seals** | 4 pieces | |

**Provided this is within the expiration date of the kit.

# OTHER SUPPLIES REQUIRED BUT NOT SUPPLIED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Pipettes and pipette tips.
3. Deionized or distilled water.
4. Squirt bottle, manifold dispenser, or automated microplate washer.
5. 500 mL graduated cylinder.

# SPECIMEN COLLECTION & STORAGE

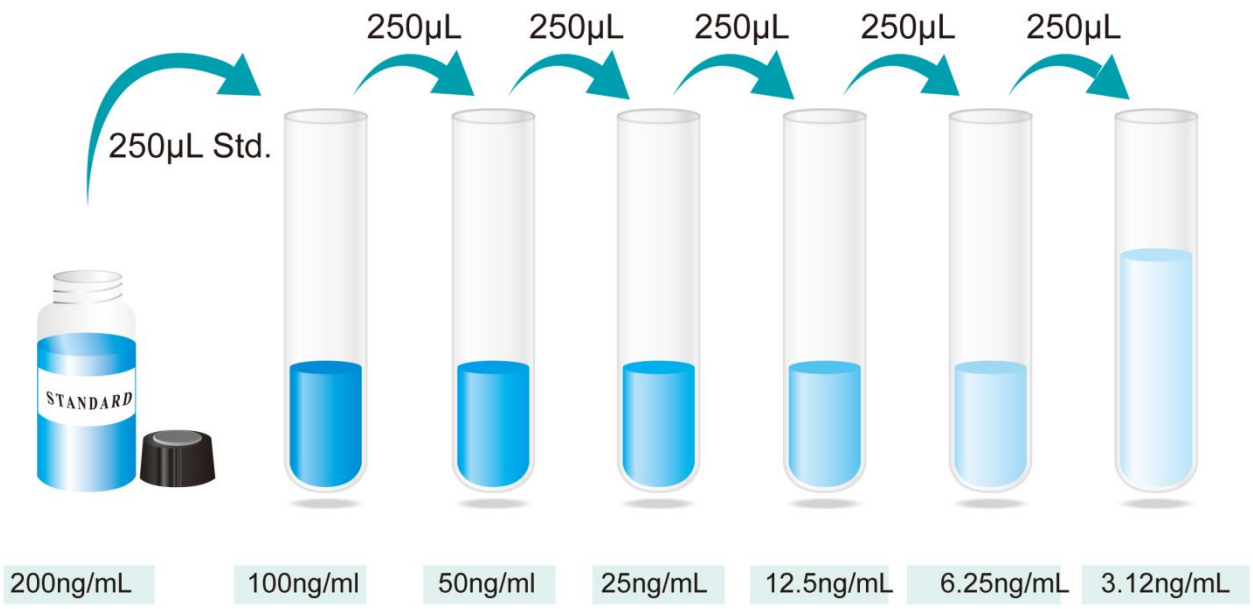
**Cell Culture Supernates** - Centrifuge cell culture media at 1000×g to remove debris. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.
**Serum** - Use a serum separator tube (SST) and allow samples to clot for 2 hours at room temperature or overnight at 2-8℃. Centrifuge at approximately for 15 minutes at 1000×g. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.
**Plasma** - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000×g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

**It is recommended to conduct a pre-test before the formal experiment to determine the dilution ratio**

# REAGENTS PREPARATION

1. **Temperature returning** - Bring all kit components and specimen to room temperature (20-25°C) before use.
2. **Wash Buffer** - Dilute 30mL of 20x Wash Buffer Concentrate with 570mL of deionized or distilled water to prepare 600mL of Wash Buffer. If crystals have formed in the concentrate Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.
3. **Standard\Sample(2 vials)** - bovine Haptoglobin Standard has a total of 2 vials. Each vial contains the standard sufficient for generating a standard curve. Reconstitute the Standard with 1.0mL of **Standard /Sample Diluent**. This reconstitution produces a stock solution of 200ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.Pipette 250μL of **Standard /Sample Diluent** into 100 ng/ml tube and the remaining tubes. Use the stock solution of 200 ng/mL to produce a 2-fold dilution series (below). Mix each tube thoroughly(vortex 20 sec for each of dilution step) and change pipette tips between each transfer. The 200 ng/mL standard serves as the high standard. The **Standard**

/**Sample Diluent** serves as the zero standard (0 ng/mL).




**Preparation of bovine Haptoglobin standard dilutions**

***If you do not run out of re-melting standard, store it at -20℃. Diluted standard shall not be reused.**

4. **Working solution of Biotin-Conjugate anti-bovine Haptoglobin antibody(1 vial)** - The lyophilized Detection Antibody should be stored at 4℃ to -20℃ in a manual defrost freezer for up to 6 months, if not used immediately. Centrifuge for 1 min at 6000 x g to bring down the material prior to open the vial. The vial contains sufficient Detection Antibody for a 96-well plate. Add **110 μL** of sterile Biotin-Conjugate antibody Diluent to each vial and vortex 30 sec to obtain the stock solution. If the entire 96-well plate is used, take **50μL** of detection antibody stock solution into **10 mL** of Biotin-Conjugate antibody Diluent to make working dilution of Detection Antibody and mix thoroughly prior to the assay. If the partial antibody is used. make a **1:200** dilution of the concentrated Biotin-Conjugate solution with the Biotin-Conjugate antibody Diluent in a clean plastic tube.

***The working solution should be used within one day after dilution.**

5. **Working solution of Streptavidin-HRP(120μL)** - Centrifuge for 1 min at 6000 x g to bring down the material prior to open the vial. The vial contains **120 μL** HRP Conjugate sufficient for a 96-well plate.Make **1:100** dilutions in Reagent Diluent. If the entire 96-well plate is used, add **100 ul** of HRP Conjugate to **10 mL** of Streptavidin-HRP Diluent to make working dilution of HRP Conjugate and mix thoroughly prior to the assay. The rest of undiluted HRP Conjugate can be stored at 4℃ for up to 6 months. DO NOT FREEZE.

***The working solution should be used within one day after dilution.**

# ASSAY PROCEDURE

Prepare all reagents and standards as directed. Wash the plate 3 times before assay.

⇩

Add 100µl standard or samples to each well, shaking with Micro-oscillator (100r/min) to incubate 60 minutes at room temperature(25±2℃).

⇩ Aspirate and wash 4 times

Add 100µl working solution of bovine -Conjugate anti-bovine Haptoglobin antibody to each well, shaking with Micro-oscillator (100r/min) to incubate 60 minutes at room temperature(25±2℃).

⇩ Aspirate and wash 4 times

Add 100µl working solution of Streptavidin-HRP to each well, shaking with Micro-oscillator (100r/min) to incubate 20 minutes at room temperature(25±2℃).

⇩ Aspirate and wash 5 times

Add 100µl Substrate solution to each well, incubate 5-20 minutes (depending on signal) at room temperature(25±2℃).Protect from light.

⇩

Add 50µl Stop solution to each well. Read at 450nm within 5 minutes.

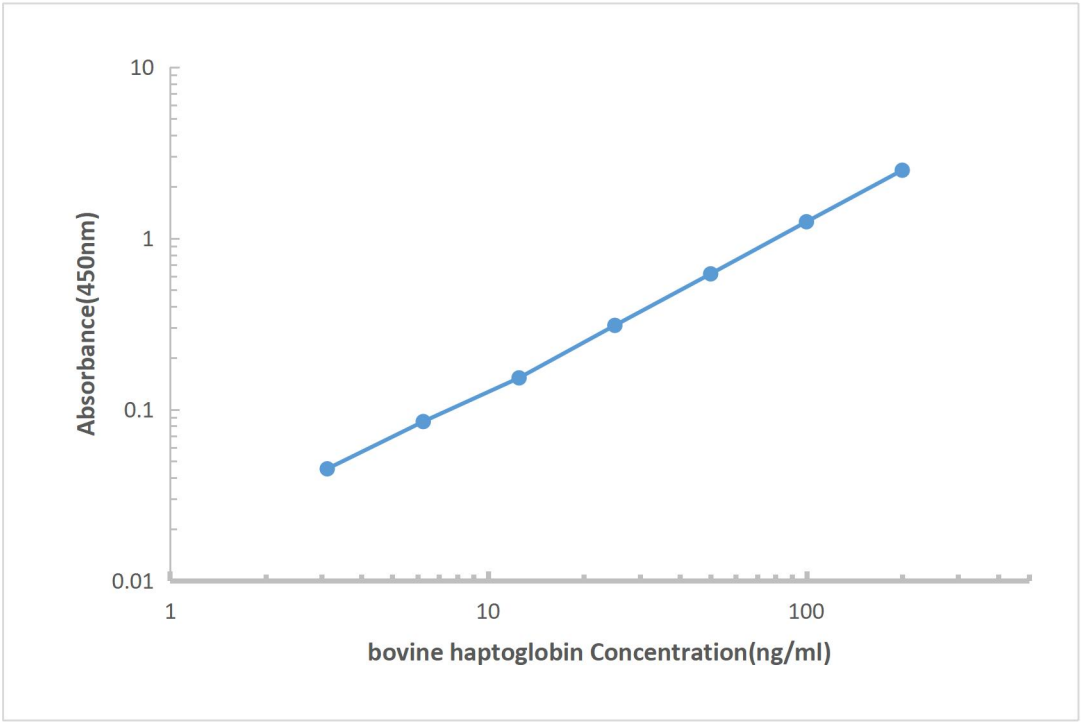
# CALCULATION OF RESULTS

1. The standard curve is used to determine the amount of specimens.
2. First, average the duplicate readings for each standard, control, and sample. All O.D. values are subtracted by the mean value of blank control before result interpretation.
3. Construct a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph.
4. The data may be linearized by plotting the log of the Haptoglobin concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

5. This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

**Typical data using the Haptoglobin ELISA**

| Std (ng/mL) | O.D.1 | O.D.2 | Averag | Correct |
|---|---|---|---|---|
| **0** | 0. 032 | 0. 035 | 0. 033 | --- |
| **3.12** | 0. 152 | 0. 153 | 0. 152 | 0. 119 |
| **6.25** | 0. 258 | 0. 262 | 0. 260 | 0. 226 |
| **12.5** | 0. 421 | 0. 441 | 0. 431 | 0. 397 |
| **25** | 0. 668 | 0. 645 | 0. 656 | 0. 623 |
| **50** | 0. 963 | 0. 986 | 0. 974 | 0. 941 |
| **100** | 1. 785 | 1. 774 | 1. 779 | 1. 746 |
| **200** | 2. 543 | 2. 471 | 2. 507 | 2. 473 |




**Representative standard curve for Haptoglobin ELISA.**

# Performance Characteristics

**SENSITIVITY:** The minimum detectable dose was 510 pg/mL.

**SPECIFICITY:** This assay recognizes both natural and recombinant bovine Haptoglobin. The factors listed below were prepared at 10ng/ml in Standard /sample Diluent and assayed for cross-reactivity and no significant cross-reactivity or interference was observed.

BMP1, BMP2, BMP4, BMP7, CRP, CCL2, CCL4, CCL5, HGF, HSP27, IGF-1, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-13, IL-15, IL-17C, IL-21, IFNγ, PDGF, PLA2G7, serpin E1, TGFβ1, TGFβ2, TGFβ3, TLR1, TLR2, TLR3, TLR9, TNF-α, TNF RI, TNF RII, VEGF,

.**REPEATABILITY:** The coefficient of variation of both intra-assay and inter-assay were less than 10%.

**RECOVERY：** The recovery of Haptoglobin spiked to three different levels in four samples throughout the range of the assay in various matrices was evaluated.

**Recovery of Haptoglobin in two matrices**

| Sample Type | Average % of Expected Range (%) | Range (%) |
|---|---|---|
| Citrate plasma | 93 | 85-102 |
| Cell culture supernatants | 96 | 86-105 |

**LINEARITY:** To assess the linearity of the assay, three samples were spiked with high concentrations of Haptoglobin in various matrices and diluted with the appropriate Sample Diluent to produce samples with values within the dynamic range of the assay.

| Dilution ratio | Recovery (%) | Citrate plasma | Cell culture supernatants |
|---|---|---|---|
| 1:2 | Average% of Expected | 94 | 104 |
| | Range (%) | 87-104 | 95-116 |
| 1:4 | Average% of Expected | 96 | 102 |
| | Range (%) | 89-108 | 97-118 |

# REFERENCES

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3. Trayhurn P, Wood IS (2004). *Br. J. Nutr.* **92** (3): 347–55.
4. Saremi B, et al. (2012). *Vet Immunol Immunopathol.* **146** (3-4): 201–11