Innovative RESEARCH Results Driven Research

Human Haptoglobulin ELISA Kit
(Plasma and Serum)

Introduction

Haptoglobulin (Hpt) is a plasma protein with hemoglobin-binding capacity, and plasma glycoproteins that form a stable complex with hemoglobin to aid the recycling of heme iron. It is a well-known marker of hemolysis (1). High Haptoglobulin level in plasma was associated with an increased cardiovascular risk in obese men (2), inflammation (3), atherosclerosis (4), and systemic sclerosis (5).

Principal of the Assay

The Human Haptoglobulin ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for detection of human Haptoglobulin in plasma and serum. This assay employs a quantitative competitive enzyme immunoassay technique that measures human Haptoglobulin in less than 2 hours. A polyclonal antibody specific for human Haptoglobulin has been pre-coated onto a 96-well microplate with removable strips. Haptoglobulin in standards and samples is competed with a biotinylated Haptoglobulin sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-protein, and SP conjugate) as instructed, prior to running the assay.**
- **Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial before opening and using contents.**
- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution.

Reagents

- **Human Haptoglobulin Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human Haptoglobulin.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human Haptoglobulin Standard:** Human Haptoglobulin in a buffered protein base (200 µg, lyophilized).

- **Biotinylated Haptoglobulin:** 1 vial, lyophilized.
- **MIX Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml, 1 bottle).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Store SP Conjugate at -20^{0}C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8^{0}C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8^{0}C.
- Store Standard and Biotinylated Protein at 2-8^{0}C before reconstituting with Diluent and at -20^{0}C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl and multiple channel).
- Deionized or distilled reagent grade water.

Sample Collection, Preparation and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes. Dilute samples 1:2000 into MIX Diluent and assay. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA or Heparin can also be used as anticoagulant.)
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum, dilute samples 1:2000 into MIX Diluent and assay. Store serum at -20^{0}C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **MIX Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8^{0}C.
- **Standard Curve:** Reconstitute the 200 µg of Haptoglobulin Standard with 5 ml of MIX Diluent to generate a stock of 40 µg/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Dilute the standard stock (40 µg/ml) 1:2 with MIX Diluent to generate a standard solution of 20 µg/ml. Prepare duplicate or triplicate standard points by serially diluting the standard solution (20 µg/ml) 1:4 with MIX Diluent to produce 5, 1.25, 0.313, and 0.078 µg/ml solutions. MIX Diluent serves as the zero standard (0 µg/ml). Any remaining solution should be frozen at -20^{0}C.

Standard Point	Dilution	[Haptoglobulin] (μg/ml)
P1	1 part Std (40 μg/ml) + 1 part MIX Diluent	20.000
P2	1 part P1 + 3 parts MIX Diluent	5.000
P3	1 part P2 + 3 parts MIX Diluent	1.250
P4	1 part P3 + 3 parts MIX Diluent	0.313
P5	1 part P4 + 3 parts MIX Diluent	0.078
P6	MIX Diluent	0.000

- **Biotinylated Haptoglobulin (4X):** Dilute Biotinylated Haptoglobulin with 4 ml MIX Diluent to produce a 4-fold stock solution. Allow to sit for 10 minutes with gentle agitation prior to making dilutions. The stock solution should be further diluted 1:4 with the MIX Diluent. Any remaining solution should be frozen at -20^{0}C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20^{0}C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 25 μl of standard or sample per well and immediately add 25 μl of Biotinylated Haptoglobulin to each well (on top of the Standard or sample) and mix gently. Cover wells with a sealing tape and incubate for one hour. Start the timer after the last sample addition.
- Wash five times with 200 μl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
  If using a machine wash six times with 300 μl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 μl of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 μl of Chromogen Substrate per well and incubate for about 7 minutes or till the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 μl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

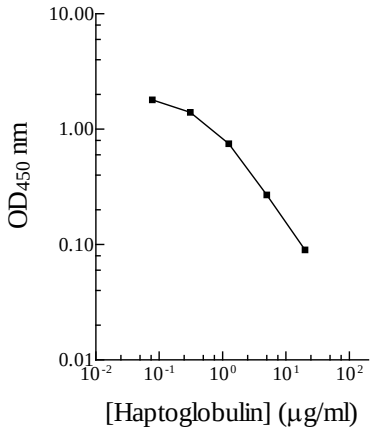
Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.

H. Haptoglobulin
Standard Curve



Performance Characteristics

- The minimum detectable dose of Haptoglobulin is ~ 0.07 µg/ml.
- Intra-assay and inter-assay coefficients of variation were 4.9% and 7.5% respectively.

Linearity

	Average Percentage of Expected Value	
Sample Dilution	**Plasma**	**Serum**
1:1000	102%	97%
1:2000	100%	99%
1:4000	104%	101%

Recovery

Standard Added Value	0.1 – 5 µg/ml
Recovery %	84-108 %
Average Recovery %	98 %

Cross-Reactivity

Species	% Cross Reactivity
Canine	None
Monkey	0.1%
Mouse	1%
Rat	0.1%
Swine	0.1%
Bovine	None
Rabbit	0.1%
Human	100%

References

(1) Van Vlierberghe H et *al* (2004) Clin *Chim Acta.* 345(1-2): 35-42
(2) Engstrom G *et al.* (2004) *Arterioscler Thromb Vasc Biol.* 24(8): 1498-502
(3) Rocha-Pereira P *et al.* (2004) *Br J Dermatol.* 150(5): 917-28
(4) Matuszek MA *et al.* (2003) *Atherosclerosis* 168(2): 389-96
(5) Kucharz EJ *et al.* (2000) Clin *Rheumatol* 19(2): 165-6