Human Hemopexin ELISA Kit
(Urine, Saliva, Milk and Cell Culture Samples)

Introduction

Hemopexin is a heme-binding plasma glycoprotein which, after haptoglobin, forms the second line of defense against hemoglobin-mediated oxidative damage during intravascular hemolysis. A decrease in plasma hemopexin concentration reflects a recent release of heme compounds in the extracellular compartment. Heme–hemopexin complexes are delivered to hepatocytes by receptor-mediated endocytosis after which hemopexin is recycled to the circulation (1). Studies indicated that increased hemopexin level associate with minimal change disease (MCD) (2), sporadic Alzheimer's disease (AD) (3), heavy-drinking chronic alcoholics (4), hemolytic anemias, chronic neuromuscular diseases and acute intermittent porphyria (5).

Principal of the Assay

The Human Hemopexin ELISA kit is designed for detection of human Hemopexin in urine, saliva, milk and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures hemopexin in less than 4 hours. A polyclonal antibody specific for hemopexin has been pre-coated onto a microplate. Hemopexin in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for hemopexin, which is recognized by a 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-antibody, 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 and the biotinylated-antibody 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

- **Hemopexin Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human hemopexin.

- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes, which can be cut to fit the format of the individual assay.
- **Hemopexin Standard:** Human Hemopexin in a buffered protein base (800 ng, lyophilized).
- **Biotinylated Hemopexin Antibody (100x):** A 100-fold concentrated biotinylated polyclonal antibody against hemopexin (80 µl).
- **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, 2 bottles).
- **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 and Biotinylated Antibody 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 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 and Storage

- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Dilute samples 1:10 into MIX Diluent. Store samples at -20^{0}C or below. Avoid repeated freeze-thaw cycles.
- **Urine:** Collect urine using sample pot. Centrifuge samples at 600 x g for 10 minutes and assay. Dilute samples 1:10 into MIX Diluent. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Saliva:** Collect saliva using sample tube. Centrifuge samples at 600 x g for 10 minutes and assay. Dilute samples 1:60 into MIX Diluent. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Milk:** Collect milk using sample tube. Centrifuge samples at 600 x g for 10 minutes and assay. Dilute samples 1:300 into MIX Diluent. Store samples at -20^{0}C or below for up to 3 months. 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.
- **Hemopexin Standard:** Reconstitute the 800 ng of human hemopexin Standard with 2 ml of MIX Diluent to generate a stock solution of 400 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the Standard solution (400 ng/ml) 1:2 with MIX Diluent to produce 200, 100, 50, 25, 12.5 and 6.25 ng/ml. Sample Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^{0}C.

Standard Point	Dilution	[Hemopexin] (ng/ml)
P1	1 part Standard (400 ng/ml)	400.00
P2	1 part P1 + 1 part MIX Diluent	200.00
P3	1 part P2 + 1 part MIX Diluent	100.00
P4	1 part P3 + 1 part MIX Diluent	50.00
P5	1 part P4 + 1 part MIX Diluent	25.00
P6	1 part P5 + 1 part MIX Diluent	12.50
P7	1 part P6 + 1 part MIX Diluent	6.25
P8	MIX Diluent	0.00

- **Biotinylated Hemopexin Antibody (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the antibody 1:100 with 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 50 µl of Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. 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.
- Add 50 µl of Biotinylated Hemopexin Antibody to each well and incubate for one hour.
- Wash the microplate as described above.
- Add 50 µl of Streptavidin-Peroxidase Conjugate per 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 10 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.

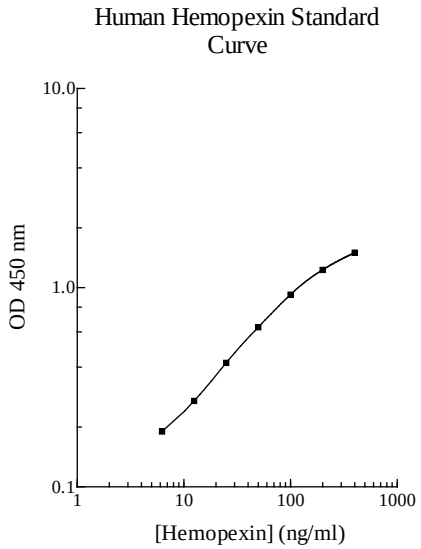
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may 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.



Performance Characteristics

- The minimum detectable dose of hemopexin is typically ~ 6 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.8 % and 7.3% respectively.

Linearity

	Average Percentage of Expected Value
Sample Dilution	**Urine**
1:5	100%
1:10	100%
1:20	101%

	Average Percentage of Expected Value
Sample Dilution	**Saliva**
1:30	97%
1:60	98%
1:120	102%

	Average Percentage of Expected Value
Sample Dilution	**Milk**
1:150	96%
1:300	100%
1:600	103%

Recovery

Standard Added Value	10 – 100 ng/ml
Recovery %	81-119 %
Average Recovery %	100 %

Cross-Reactivity

Species	% Cross Reactivity
Canine	< 4 (Suggest dilution 1:400 for plasma)
Monkey	> 40
Mouse	<5 (Suggest dilution 1:400 for plasma)
Rat	<2 (Suggest dilution 1:400 for plasma)
Swine	<5 (suggest dilution 1:400 for plasma)