

## Human Haptoglobin ELISA Kit

**Cat#: orb507543 (ELISA Manual)**

### Assay Principle

The Biorbyt Human HP Pre-Coated ELISA (Enzyme-Linked Immunosorbent Assay) kit is a solid-phase immunoassay specially designed to measure Human HP with a 96-well strip plate that is pre-coated with antibody specific for HP. The detection antibody is a biotinylated antibody specific for HP. The capture antibody is a monoclonal antibody from mouse, the detection antibody is a biotinylated polyclonal antibody from goat. The kit contains recombinant Human HP with immunogen: Expression system for standard: NSO, Immunogen sequence: V19-N406 . The kit is analytically validated with ready-to-use reagents. To measure Human HP, add standards and samples to the wells, then add the biotinylated detection antibody. Wash the wells with PBS or TBS buffer, and add Avidin-Biotin-Peroxidase Complex (ABC-HRP). Wash away the unbound ABC-HRP with PBS or TBS buffer and add TMB. TMB is an HRP substrate and will be catalyzed to produce a blue color product, which changes into yellow after adding the acidic stop solution. The density of the yellow product is linearly proportional to Human HP in the sample. Read the density of the yellow product in each well using a plate reader, and benchmark the sample wells' readings against the standard curve to determine the concentration of Human HP in the sample.

### Overview

Product Name Human Haptoglobin ELISA Kit

Reactive Species Human

Size 96wells/kit, with removable strips.

Description Sandwich High Sensitivity ELISA kit for Quantitative Detection of human Haptoglobin.

96wells/kit, with removable strips.

Sensitivity <20pg/ml

\*The sensitivity or the minimum detectable dose (MDD) is the lower limit of the target protein that can be detected by the kit. It is determined by adding two standard deviations to the mean O.D. value of twenty (20) blank wells and calculating the corresponding concentration.

Detection Range 312pg/ml-20000pg/ml

Storage Instructions Store at 4°C for 6 months, at -20°C for 13 months. Avoid multiple freeze-thaw cycles (Shipped with wet ice.)

Uniprot ID P00738

### Technical Details

Capture/Detection Antibodies

The capture antibody is a monoclonal antibody from mouse, the detection antibody is a biotinylated polyclonal antibody from goat.

## Specificity

Natural and recombinant Human HP

## Immunogen

Expression system for standard: NSO, Immunogen sequence: V19-N406

## Cross Reactivity

This kit is for the detection of Human HP. No significant cross-reactivity or interference between HP and its analogs was observed. This claim is limited by existing techniques therefore cross-reactivity may exist with untested analogs.

## Preparations Before Assay

Please read the following instructions before starting the experiment.

1. Read this manual in its entirety in order to minimize the chance of error.
2. Confirm that you have the appropriate non-supplied equipment available.
3. Confirm that the species, target antigen, and sensitivity of this kit are appropriate for your intended application.
4. Confirm that your samples have been prepared appropriately based upon recommendations (see Sample Preparation) and that you have sufficient sample volume for use in the assay.
5. When first using a kit, appropriate validation steps should be taken before using valuable samples. Confirm that the kit adequately detects the target antigen in your intended sample type(s) by running control samples.
6. If the concentration of target antigen within your samples is unknown, a preliminary experiment should be run using a control sample to determine the optimal sample dilution (see Sample Preparation).
7. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, a pilot experiment using standards and a small number of samples is recommended.
8. Before using the kit, spin tubes to bring down all components to the bottom of the tubes.
9. Don't let the 96-well plate dry out since this will inactivate active components on the plate.
10. Don't reuse tips and tubes to avoid-cross contamination.
11. Avoid using the reagents from different batches together.
12. The kit should not be used beyond the expiration date on the kit label. Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding. Variations in sample collection, processing, and storage may cause sample value differences.

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## Kit Components/Materials Provided

Description	Quantity	Volume	Storage of opened/reconstituted material
Anti-Human HP Pre-coated 96-well Strip Microplate	1	12 strips of 8 wells	Return unused wells to the foil pouch. Reseal along the entire edge of the zip-seal. May be stored for up to 1 month at 4°C provided this is within the expiration date of the kit.
Human HP Standard	2	200ng/tube	Discard the HP stock solution after 12 hours at 4°C. May be stored at -20°C for 48 hours.
Human HP Biotinylated Antibody (100x)	1	100 µl	May be stored for up to 1 month at 4°C provided this is within the expiration date of the kit.
Avidin-Biotin-Peroxidase Complex (100x)	1	100 µl	
Sample Diluent	1	30 ml	
Antibody Diluent	1	12 ml	
Avidin-Biotin-Peroxidase Diluent	1	12 ml	
Color Developing Reagent (TMB)	1	10 ml	
Stop Solution	1	10 ml	
Wash Buffer (25x)	1	20 ml	
Plate Sealers	4	Piece	

## Required Materials That Are Not Supplied

Microplate reader capable of reading absorbance at 450nm.

Automated plate washer (optional)

Pipettes and pipette tips capable of precisely dispensing 0.5 µl through 1 ml volumes of aqueous solutions.

Multichannel pipettes are recommended for a large number of samples.

Deionized or distilled water.

500ml graduated cylinders.

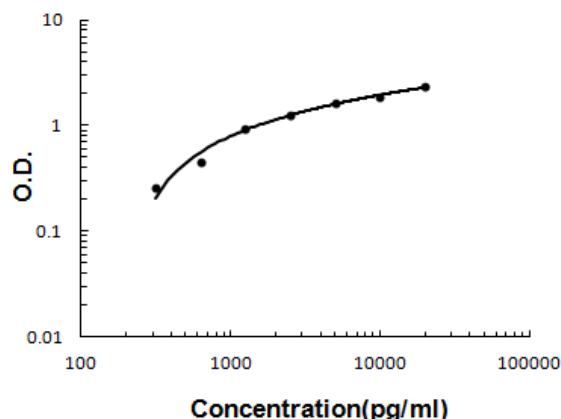
Test tubes for dilution.

## Human Haptoglobin ELISA Kit Standard Curve Example

The highest O.D. value might be higher or lower than in the example. The experiment result is statistically significant if the highest O.D. value is no less than 1.0.

Concentration0 (pg/ml)	312	625	1250	2500	5000	10000	20000	
O.D.	0.070	0.332	0.526	0.998	1.329	1.720	1.961	2.389

### Human Haptoglobin ELISA Kit standard curve Human Haptoglobin ELISA Kit



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

#### Intra/Inter Assay Variability

Biorbyt spends great efforts in documenting lot-to-lot variability and ensuring our assay kits produce robust data that are reproducible.

**Intra-Assay Precision (Precision within an assay):** Three samples of known concentration were tested on one plate to assess intra-assay precision.

**Inter-Assay Precision (Precision across assays):** Three samples of known concentration were tested in separate assays to assess interassay precision.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	16	16	16	24	24	24
Mean(pg/ml)	778	2764	9900	774	2895	9099
Standard deviation	37.34	110.56	712.8	39.47	167.91	718.82
CV(%)	4.8%	4%	7.2%	7.9%	5.8%	7.9%

#### Reproducibility

To assay reproducibility, three samples with differing target protein concentrations were assayed using four different lots.

Lots	Lot1 (pg/ml)	Lot2 (pg/ml)	Lot3 (pg/ml)	Lot4 (pg/ml)	Mean (pg/ml)	Standard Deviation	CV (%)
Sample 1	778	698	804	781	765	40.1	5.2%
Sample 2	2764	2697	2705	2507	2668	96.62	3.6%
Sample 3	9900	10211	10114	10441	10166	194.36	1.9%

\*number of samples for each test n=16.

## Preparation Before The Experiment

### All reagents

Bring all reagents to room temperature (18-25°C) prior to use. Please DO NOT equilibrate unused plate well strips to room temperature. They should be sealed and stored in the original packaging. We recommend doing it at 37°C for best consistency with our QC results. Also, the TMB incubation time estimate (15-25 min) is based on incubation at 37°C.

### Wash buffer

Prepare 500 ml of Working Wash Buffer by diluting the supplied 20 ml of Wash Buffer (25 x) with 480 ml of deionized or distilled water. If crystals have formed in the concentrate, warm to room temperature and mix it gently until crystals have completely dissolved.

### Biotinylated Anti-Human HP antibody

It is recommended to prepare this reagent immediately prior to use by diluting the Human HP Biotinylated antibody (100x) 1:100 with Antibody Diluent. Prepare 100 µl by adding 1 µl of Biotinylated antibody (100x) to 99 µl of Antibody Diluent for each well. Mix gently and thoroughly and use within 2 hours of generation.

### Avidin-Biotin-Peroxidase Complex

It is recommended to prepare this reagent immediately prior to use by diluting the Avidin-Biotin-Peroxidase Complex (100x) 1:100 with Avidin-Biotin-Peroxidase Diluent. Prepare 100 µl by adding 1 µl of Avidin-Biotin-Peroxidase Complex (100x) to 99 µl of Avidin-Biotin-Peroxidase Diluent for each well. Mix gently and thoroughly and use within 2 hours of generation.

### Human HP Standard

It is recommended that the standards be prepared no more than 2 hours prior to performing the experiment. Use one 200ng of lyophilized Human HP standard for each experiment. Gently spin the vial prior to use. Reconstitute the standard to a stock concentration of 200ng/ml using 1ml of sample diluent. Allow the standard to sit for a minimum of 10 minutes with gentle agitation prior to making dilutions.

**Microplate** The included microplate is coated with capture antibodies and is ready-to-use. It does not require additional washing or blocking. The unused well strips should be sealed and stored in the original packaging.

## Dilution of Human HP Standard

1. Number tubes 1-8. Final Concentrations to be Tube # 1 –20000pg/ml, #2 –10000pg/ml, #3 – 5000pg/ml, #4 – 2500pg/ml, #5 – 1250pg/ml, #6 – 625pg/ml, #7 – 312.5pg/ml, #8 – Sample Diluent serves as the zero standard (0pg/ml).
2. To generate standard #1, add 100µl of the reconstituted standard stock solution of 200ng/ml and 900µl of sample diluent to tube #1 for a final volume of 1000µl. Mix thoroughly.
3. Add 300 µl of sample diluent to tubes # 2-7.
4. To generate standard #2, add 300 µl of standard #1 from tube #1 to tube #2 for a final volume of 600 µl. Mix thoroughly.

5. To generate standard #3, add 300  $\mu$ l of standard #2 from tube #2 to tube #3 for a final volume of 600  $\mu$ l. Mix thoroughly.
6. Continue the serial dilution for tube #4-7.

### Sample Preparation and Storage

These sample collection instructions and storage conditions are intended as a general guideline, and the sample stability has not been evaluated.

#### Cell culture supernatants

Clear sample of particulates by centrifugation, assay immediately or store samples at -20°C.

#### Serum

Use a serum separator tube (SST) and allow serum to clot at room temperature for about four hours. Then, centrifuge for 15 min at approximately 1,000 x g. assay immediately or store samples at -20°C.

#### Plasma

Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 min at approximately 1,000 x g. Assay immediately or store samples at -20°C.

\*Note: it is important to not use anticoagulants other than the ones described above to treat plasma for other anticoagulants could block the antibody binding site.

### Sample Collection Notes

1. Biorbyt recommends that samples are used immediately upon preparation.
2. Avoid repeated freeze/thaw cycles for all samples.
3. In the event that a sample type not listed above is intended to be used with the kit, it is recommended that the customer conduct validation experiments in order to be confident in the results.
4. Due to chemical interference, the use of tissue or cell extraction samples prepared by chemical lysis buffers may result in inaccurate results.
5. Due to factors including cell viability, cell number, or sampling time, samples from cell culture supernatant may not be detected by the kit.
6. Samples should be brought to room temperature (18-25°C) before performing the assay without the use of extra heating.
7. Sample concentrations should be predicted before being used in the assay. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
8. Biorbyt is responsible for the quality and performance of the kit components but is NOT responsible for the performance of customer supplied samples used with the kit.

## Sample Dilution Guideline

The user needs to estimate the concentration of the target protein in the sample and use an appropriate dilution factor so that the diluted target protein concentration falls in the range of O.D. values of the standard curve. Dilute the sample using provided diluent buffer. Pilot tests using a dilution series of each sample type are necessary. The sample must be mixed thoroughly with Sample Diluent.

## Assay Protocol

It is recommended that all reagents and materials be equilibrated to room temperature (18-25°C) prior to the experiment (see Preparation Before The Experiment, if you have missed this information).

1. Prepare all reagents and working standards as directed previously.
2. Remove excess microplate strips from the plate frame and seal and store them in the original packaging.
3. Add 100 µl of the standard, samples, or control per well. Add 100 µl of the **Sample Diluent** into the zero well. At least two replicates of each standard, sample, or control is recommended.
4. Cover with the plate sealer provided and incubate for 120 minutes at room temperature (or 90 min. at 37 °C).
5. Remove the cover and discard the liquid in the wells into an appropriate waste receptacle. Invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
6. Add 100 µl of the prepared **1x Biotinylated Anti-Human HP antibody** to each well.
7. Cover with a plate sealer and incubate for 90 minutes at room temperature (or 60 minutes at 37°C).
8. Wash the plate 3 times with the **1x wash buffer**:
  - a. Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
  - b. Add 300 µl of the **1x wash buffer** to each assay well. (For cleaner background incubate for 60 seconds between each wash).
  - c. Repeat steps a-b 2 additional times.
9. Add 100 µl of the prepared **1x Avidin-Biotin-Peroxidase Complex** into each well. Cover with the plate sealer provided and incubate for 40 minutes at RT (or 30 minutes at 37°C).
10. Wash the plate 5 times with the **1x wash buffer**:
  - a. Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
  - b. Add 300 µl of the **1x wash buffer** to each assay well. (For cleaner background incubate for 60 seconds between each wash).
  - c. Repeat steps a-b 4 additional times.
11. Add 90 µl of **Color Developing Reagent** to each well. Cover with the plate sealer provided and incubate in the dark for 30 minutes at RT (or 15-25 minutes at 37°C). (The optimal incubation time must be empirically determined. A guideline to look for is blue shading the top four standard wells, while the remaining standards remain clear.)
12. Add 100 µl of **Stop Solution** to each well. The color should immediately change to yellow.

13. Within 30 minutes of stopping the reaction, the O.D. absorbance should be read with a microplate reader at 450nm.

### Assay Protocol Notes

1. Solutions: To avoid cross-contamination, change pipette tips between additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

2. Applying Solutions: All solutions should be added to the bottom of the ELISA plate well. Avoid touching the inside wall of the well. Avoid foaming when possible.

3. Assay Timing: The interval between adding samples to the first and last wells should be minimized. Delays will increase the incubation time differential between wells, which will significantly affect the experimental accuracy and repeatability. For each step in the procedure, total dispensing time for addition of reagents or samples should not exceed 10 minutes.

4. Incubation: To prevent evaporation and ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods of time between incubation steps. Do not let wells dry out at any time during the assay. Strictly observe the recommended incubation times and temperatures.

5. Washing: Proper washing procedure is critical. Insufficient washing will result in poor precision and falsely elevated absorbance readings.

Residual liquid in the reaction wells should be patted dry against absorbent paper during the washing process. Do not put absorbent paper directly into the reaction wells.

6. Controlling Substrate Reaction Time: After the addition of the TMB Substrate, periodically monitor the color development. Stop color development before the color becomes too deep by adding Stop Solution. The excessively strong color will result in inaccurate absorbance readings.

7. Reading: The microplate reader should be preheated and programmed prior to use. Prior to taking OD readings, remove any residual liquid or fingerprints from the underside of the plate and confirm that there are no bubbles in the wells.

8. Reaction Time Control: Control reaction time should be strictly followed as outlined.

9. Stop Solution: The Stop Solution contains an acid, therefore proper precautions should be taken during its use, such as protection of the eyes, hands, face, and clothing.

10. To minimize the external influence on the assay performance, operational procedures and lab conditions (such as room temperature, humidity, incubator temperature) should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

### Data Analysis

Average the duplicate readings for each standard, sample, and control. Subtract the average zero standard O.D. reading. It is recommended that a standard curve be created using computer software to generate a four-parameter logistic (4-PL) curve-fit. Alternatively, plot the mean absorbance for each standard against the concentration. The measured concentration in the sample can be interpolated by using linear regression of each average relative OD against the standard curve generated using curve fitting software. This will generate an adequate but less precise fit of the data. For diluted samples, the concentration reading from the standard curve must be multiplied by the dilution factor.



## Background on HP

Haptoglobin (HP), is a protein that in humans is encoded by the HP gene. Haptoglobin, a plasma glycoprotein that binds free hemoglobin, has a tetrameric structure of 2 alpha and 2 beta polypeptides that are covalently associated by disulfide bonds. Haptoglobin is homologous to serine proteases of the chymotrypsinogen family. In the chimpanzee, there are 3 genes in the haptoglobin family (haptoglobin, HP; haptoglobin-related, HPR; and haptoglobin-primate, HPP), whereas only 2 genes exist in humans (HP and HPR). There are similarities between the primary structures of the alpha chain and of light chains of gamma globulins. The alpha haptoglobin locus is on the long arm of chromosome 16, where its exact cytogenetic location is 16q22.2. A major function of haptoglobin is to bind hemoglobin (Hb) to form a stable Hp-Hb complex and thereby prevent Hb-induced oxidative tissue damage. Haptoglobin is an unusual secretory protein in that it is proteolytically processed in the endoplasmic reticulum and not in the Golgi. The human haptoglobin HP\*2 allele contains a 1.7-kb intragenic duplication that arose after a unique nonhomologous recombination between the prototype HP\*1 alleles.

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