



# HP (Human) ELISA Kit

Catalog Number KA0533

96 assays

Version: 05

Intended for research use only

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## **Introduction**

### **Intended Use**

The HP (Human) ELISA Kit is to be used for the in vitro quantitative determination of human Haptoglobin in human serum, human plasma, cell lysate and buffered solution. The assay will recognize both native and recombinant human Haptoglobin.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

### **Background**

Haptoglobin (abbreviated as Hp) is a protein in the blood plasma that binds free hemoglobin released from erythrocytes with high affinity and removes it from the circulation to prevent kidney injury, and iron loss following hemolysis. Also, haptoglobin prevents bacteria from using the iron present in hemoglobin to grow, regulates the activity of many cell types of the immune system, and is an extracellular chaperone. Haptoglobin, as an antioxidant, has antibacterial activity and plays a role in modulating many aspects of the acute phase response. In addition, haptoglobin acts as a potent immunosuppressor of lymphocyte function and modulates the helper T-cell type 1 and type 2 (Th1/Th2) balance within the body. Haptoglobin plays a role in stimulation of angiogenesis and has highly potent cholesterol crystallization-promoting activity, also. Changes in the measured concentrations of haptoglobin in serum may help to assess the disease status of patients with inflammations, infections, malignancy etc. (increases) as well as in haemolytic conditions (decreases).

Haptoglobin in its simplest form consists of two  $\alpha$ - and two  $\beta$ -chains, connected by disulfide bridges. The chains originate from a common precursor protein which is proteolytically cleaved during protein synthesis. Haptoglobin exists in two allelic forms in the human population, so called Hp1 and Hp2; the latter one having arisen due to the partial duplication of Hp1 gene. Three phenotypes of haptoglobin therefore are found in humans: Hp1-1, Hp2-1, and Hp2-2. Hp of different phenotypes have been shown to bind hemoglobin with different affinities. Hp 1-1 is biologically the most effective in binding free hemoglobin and suppressing inflammatory responses associated with free hemoglobin. Hp 2-2 is biologically the least active, and Hp 2-1 is moderately active. The possible association of allelic polymorphism of haptoglobin with various pathologic conditions such as cardiovascular disease, autoimmune disorders, malignancy has been studied. Hp 2-2 phenotype presents characteristics of being an independent risk factor for cardiovascular disease (CVD) and more significant in diabetic CVD. In chronic kidney disease (CKD), due to various processes that take place simultaneously, the combined effect is different in the elderly and the young. The functional differences between the Haptoglobin phenotypes are most probably due to their antioxidative property and to macrophage activation with differential release of cytokines.

### **Principle of the Assay**

The design of this assay is based on a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). The microtiter plate provided in this kit has been pre-coated with a monoclonal antibody specific to human Haptoglobin. Samples are pipetted into these wells. Nonbound Haptoglobin and other components of the sample should be removed by washing, then biotin-conjugated monoclonal antibody specific to Haptoglobin added. In order to quantitatively determine the amount of Haptoglobin present in the sample, Avidin conjugated to Horseradish Peroxidase (HRP) should be added to each microplate well. The final step, a TMB-substrate solution added to each well. Finally, a sulfuric acid solution is added and the resulting yellow colored product is measured at 450 nm. Since the increases in absorbency is directly proportional to the amount of captured Haptoglobin.

## General Information

### Materials Supplied

List of component

Component	Amount
96 Well Plate (in a aluminium foil bag with desiccant): Human Haptoglobin microtiter plate, one plate of 96 wells (8 well strip x 12). A plate using break-apart strips coated with a mouse monoclonal antibody specific to human Haptoglobin beta chain.	96 wells
Washing Buffer (20x)	25 mL x 2
Standard Protein (lyophilized): Lyophilized recombinant human Haptoglobin.	1 Glass vial
Standard/Sample Dilution Buffer	25 mL
Secondary Antibody (lyophilized): Biotinylated anti human Haptoglobin beta chain.	1 Glass vial
AV-HRP (100x): Avidin linked Horseradish Peroxidase (HRP, enzyme)	150 µL
Secondary Antibody/AV-HRP Dilution Buffer	25 mL
Substrate (TMB) (Stabilized chromogen): Tetramethylbenzidine (TMB) solution	15 mL
Stop Solution: 1 N solution of sulfuric acid (H <sub>2</sub> SO <sub>4</sub> )	15 mL
Plate sealers: Adhesive sheet	2 slides

*Note: Do not mix or interchange different reagents from various kit lots.*

### Storage Instruction

All kit components of this kit are stable at 2 to 8°C. Any unused reconstituted standard should be discarded or frozen at -70°C. Standard can be frozen and thawed one time only without loss of immunoreactivity.

### Materials Required but Not Supplied

- ✓ Microtiter plate reader capable of measurement at or near 450 nm
- ✓ Calibrated, adjustable precision pipettes, preferably with disposable plastic tips (A manifold multi-channel pipette is desirable for large assays.)
- ✓ Distilled or deionized water
- ✓ Data analysis and graphing software
- ✓ Vortex mixer
- ✓ Polypropylene tubes for diluting and aliquoting standard
- ✓ Absorbent paper towels
- ✓ Calibrated beakers and graduated cylinders of various sizes

**Precautions for Use**

For research use only. Not for diagnostic or therapeutic purposes.

- ✓ Stop solution: This reagent is an irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothing. Wear suitable protective clothing, gloves and eye protection. In the event of contact with eyes or skin, wash immediately with plenty of water.
- ✓ Standard protein and 2<sup>nd</sup> Antibody containing Sodium Azide as a preservative.

## Assay Protocol

### Reagent Preparation

- Human Haptoglobin standard
  1. Reconstitute the lyophilized human haptoglobin standard by adding 1 mL of Standard/Sample Dilution Buffer to make the 1,000 ng/mL standard stock solution. Allow solution to sit at RT for 5 minutes, then gently vortex to mix completely. Use within one hour of reconstituting.
  2. Prepare 1 mL of 50 ng/mL top standard by adding 50  $\mu$ L of the above stock solution in 950  $\mu$ L of Standard/Sample Dilution Buffer. Perform 2-fold serial dilution of the top standard to make the standard curve within the range of this assay (0.78 ng/mL ~ 50 ng/mL) as below. Standard/Sample Dilution Buffer serves as the zero standard (0 ng/mL).

Standard	Add	Into
50 ng/mL	50 $\mu$ L of the std. (1,000 ng/mL)	950.0 $\mu$ L of the Standard/Sample Dilution Buffer
25 ng/mL	500 $\mu$ L of the std. (50 ng/mL)	500.0 $\mu$ L of the Standard/Sample Dilution Buffer
12.5 ng/mL	500 $\mu$ L of the std. (25 ng/mL)	500.0 $\mu$ L of the Standard/Sample Dilution Buffer
6.25 ng/mL	500 $\mu$ L of the std. (12.5 ng/mL)	500.0 $\mu$ L of the Standard/Sample Dilution Buffer
3.13 ng/mL	500 $\mu$ L of the std. (6.25 ng/mL)	500.0 $\mu$ L of the Standard/Sample Dilution Buffer
1.56 ng/mL	500 $\mu$ L of the std. (3.13 ng/mL)	500.0 $\mu$ L of the Standard/Sample Dilution Buffer
0.78 ng/mL	500 $\mu$ L of the std. (1.56 ng/mL)	500.0 $\mu$ L of the Standard/Sample Dilution Buffer
0 ng/mL	1.0 mL of the Standard/Sample Dilution Buffer	

- Secondary Antibody
 

100X secondary antibody solution can be made by adding 150  $\mu$ L secondary antibody/AV-HRP dilution buffer in the vial.

  1. Equilibrate to room temperature, mix gently.
  2. Mix 20  $\mu$ L Secondary Antibody concentrated solution (100X) + 1.98 mL Secondary Antibody/AV-HRP dilution buffer. (Sufficient for two 8-well strips, prepare more if necessary).  
Label as "Working Secondary antibody Solution".
  3. Return the unused Secondary Antibody concentrated solution to the refrigerator.
- AV-HRP
  1. Equilibrate to room temperature, mix gently.
  2. Mix 20  $\mu$ L AV-HRP concentrated solution (100X) + 1.98 mL Secondary Antibody/AV-HRP dilution buffer. (Sufficient for two 8-well strips, prepare more if needed).  
Label as "Working AV-HRP Solution".
  3. Return the unused AV-HRP concentrated solution to the refrigerator.

- Washing buffer
  1. Equilibrate to room temperature, mix to re-dissolve any precipitated salt.
  2. Mix 0.5 volume Wash buffer concentrate solution (20X) + 9.5 volumes of deionized water. Label as "Working Washing Solution".
  3. Store both the concentrated and the Working Washing Solution in the refrigerator.

*Note: \* Directions for washing*

1. *Fill the wells with 300  $\mu$ L of "Working Washing Buffer".  
Let soak for 1 to 3 minutes and then all residual wash-liquid must be drained from the wells by aspiration (taking care not to scratch the inside of the well) or decantation, followed by forceful tapping of the plate on absorbent paper. Never insert absorbent paper directly into the wells.  
If using an automated washer, the operating instructions for washing equipment should be carefully followed.*
2. *Incomplete washing will adversely affects the assay and renders false results.*
3. *It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing to avoid strips coming free of the frame.*

### **Sample Preparation**

Blood should be collected by veinpuncture. For plasma samples, blood may be drawn into tubes containing sodium citrate or heparin, EDTA. The serum or plasma should be separated from the coagulated or packed cells by centrifugation. Specimens may be shipped at room temperature and then stored refrigerated at 2-8°C if testing is to take place within one week after collection. If testing is to take place later than one week, specimens should be stored at -20°C. Avoid repeated freeze/thawing.

### **Assay Procedure**

- ✓ Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.
  - ✓ All standards, controls and samples should be run in duplicate for confirmation of reproducibility.
  - ✓ A standard curve must be run with each assay.
  - ✓ If particulate matter is present in the analyte, centrifuge or filter prior to analysis.
  - ✓ Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
1. Determine the number of 8-well strips needed for assay. Insert these in the frame(s) for current use (Re-bag extra strips and frame. Refrigerate for further use).
  2. For the standard curve, add 100  $\mu$ L of the standard to the appropriate microtiter wells. Add 100  $\mu$ L of the Standard/Sample Dilution Buffer to zero wells.
  3. Serum requires at least 10000 fold dilution in the Standard/Sample Dilution Buffer. And add 100  $\mu$ L of samples to each well.



4. Cover the plate with the plate cover and incubate for 2 hours at room temperature.
5. Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
6. Pipette 100  $\mu$ L of “Working Secondary Antibody Solution” into each well.
7. Cover the plate with the plate cover and incubate for 1 hour at room temperature.
8. Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
9. Add 100  $\mu$ L “Working AV-HRP Solution” to each well.
10. Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
11. Thoroughly aspirate or decant the solution from the wells. Wash the wells 3 times (See “Directions for washing”).
12. Add 100  $\mu$ L of Substrate to each well. The liquid in the wells should begin to turn blue.
13. Incubate the plate at room temperature.
  - ✓ Do not cover the plate with aluminum foil, or color may develop.

The incubation time for chromogen substrate is often determined by the microtiter plate reader used. O.D. values should be monitored and the substrate reaction stopped before O.D. of the positive wells exceeds the limits of the instrument. O.D. values at 450 nm can only be read after the Stop Solution has been added to each well.
  - ✓ Because the Substrate is light sensitive, avoid the remained Substrate solution prolonged exposure to light.
  - ✓ Typically, reaction is stopped 10~15 minutes after treatment of Substrate, but this time can be adjusted as the user desires.
14. Add 100  $\mu$ L of Stop Solution to each well. The solution in the wells should change from blue to yellow.
15. Read the absorbance of each well at 450 nm. Read the plate within 20 minutes of adding the Stop Solution.
16. Plot on graph paper the absorbance of the standard against the standard concentration (Optimally, the background absorbance can be subtracted from all data points, including standards, unknowns and controls, prior to plotting.). Draw a smooth curve through these points to construct the standard curve.
17. Read the human Haptoglobin concentrations for the unknown samples and controls from the standard curve plotted in step 16. Multiply value(s) obtained for the unknown sample by the dilution factor (Samples producing signals greater than that of the highest standard should be further diluted in the Standard/Sample Dilution Buffer).

## Data Analysis

### Calculation of Results

- Typical result

The standard curve below is for illustration only and should not be used to calculate results in your assay.

A standard curve must be run with each assay.

Standard Human Haptoglobin (ng/mL)	Optical Density (at 450 nm)
0	0.069
0.78	0.091
1.56	0.124
3.13	0.189
6.25	0.295
12.50	0.496
25.00	0.931
50.00	1.765

- Limitation

- ✓ Do not extrapolate the standard curve beyond the 50 ng/mL standard point.
- ✓ Other buffers and matrices have not been investigated.
- ✓ The rate of degradation of native human Haptoglobin in various matrices has not been investigated.

### Performance Characteristics

- Sensitivity

The minimal detectable dose of human Haptoglobin was calculated to be 0.78 ng/mL, by subtracting two standard deviations from the mean of 10 zero standard replicates (ELISA buffer, S0) and intersecting this value with the standard curve obtained in the same calculation.

- Specificity

The following substances have been tested and found to have no cross-reactivity: human Albumin, IgG, Transferrin, Fibrinogen,  $\alpha$ -2 macroglobulin, Complement C3, Antitrypsin.

- Precision

- Within-Run (Intra-Assay)

Mean (ng/mL)	SD (ng/mL)	CV (%)
3.56	0.34	9.5
6.15	0.49	6.3
13.02	0.57	3.6
25.40	1.01	4.0

(n=5)

- Between-Run (Inter-Assay)

Mean (ng/mL)	SD (ng/mL)	CV (%)
3.45	0.19	5.4
6.39	0.28	4.3
12.75	0.59	4.6
24.79	0.93	3.8

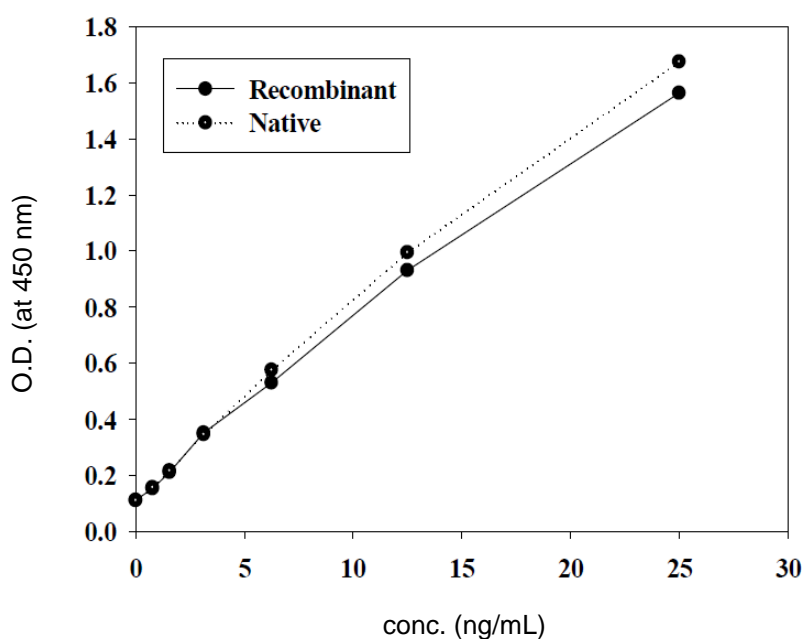
(n=11)

- Recovery

Recovery on addition is 98.3~101.5% (mean 99.9%)

Recovery on dilution is 96.2~104.8% (mean 101.0%)

- Parallelism



Native human Haptoglobin from human serum was serially diluted in sample dilution buffer. The optical density of each dilution was plotted against the recombinant human Haptoglobin standard curve.

## Resources

### Troubleshooting

Problem	Possible Cause	Solution
High signal and background in all wells	• Insufficient washing	• Increase number of washes • Increase time of soaking between in wash
	• Too much Streptavidin-HRP	• Check dilution, titration
	• Incubation time too long	• Reduce incubation time
	• Development time too long	• Decrease the incubation time before the stop solution is added
No signal	• Reagent added in incorrect order, or incorrectly prepared	• Review protocol
	• Standard has gone bad (If there is a signal in the sample wells)	• Check the condition of stored standard
	• Assay was conducted from an incorrect starting point	• Reagents allows to come to 20~30°C before performing assay
Too much signal – whole plate turned uniformly blue	• Insufficient washing – unbound SAV-HRP remaining	• Increase number of washes carefully
	• Too much Streptavidin-HRP	• Check dilution
	• Plate sealer or reservoir reused, resulting in presence of residual Streptavidin-HRP	• Use fresh plate sealer and reagent reservoir for each step
Standard curve achieved but poor discrimination between point	• Plate not developed long enough	• Increase substrate solution incubation time
	• Improper calculation of standard curve dilution	• Check dilution, make new standard curve
No signal when a signal is expected, but standard curve looks fine	• Sample matrix is masking detection	• More diluted sample recommended
Samples are reading too high, but standard curve is fine	• Samples contain protein levels above assay range	• Dilute samples and run again
Edge effect	• Uneven temperature around work surface	• Avoid incubating plate in areas where environmental conditions vary • Use plate sealer

### **References**

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3. Dobryszczycka W. Eur J Clin Chem Clin Biochem. 1997; vol.35(9): pp.647-54.

**Plate Layout**

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H