Haptoglobin (Hp) Typing ELISA

Enzyme-Linked Immunosorbent Assay (ELISA) for qualitative determination of Haptoglobin phenotypes (Hp 1-1, Hp 2-1, or Hp 2-2) in human serum/plasma.

Instruction Manual

Test kit for 96 determinations
Catalog Number: 710-01U

For Research Use Only

Store at 2-8°C (35.6º-46.4ºF)**.**
Do Not Freeze

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Intended Use

The Haptoglobin typing test is an Enzyme-Linked Immunosorbent Assay (ELISA) for qualitative determination of Haptoglobin phenotypes (Hp 1-1, Hp 2-1, or Hp 2-2) in human serum/plasma.

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Introduction

Haptoglobin (Hp) is a normally occurring acute phase serum protein whose primary physiological role is to scavenge free hemoglobin (Hb), a potent oxidizing agent, from the circulation (1). Free Hb, released during hemolysis of red blood cells, promotes the accumulation of hydroxyl free radicals which can cause oxidative damage to tissues. Hp acts as an antioxidant by first forming complexes with Hb and then clearing the complexes from the circulation by uptake via the CD163 macrophage receptor (2).

Hp is polymorphic in man and occurs as either one of three phenotypes, Hp 1-1, Hp 2-1, or Hp 2-2. The prevalence of the three phenotypes of Hp is 16% Hp 1-1, 48% Hp 2-1, and 36% Hp 2-2 (1). Substantial evidence supports the pathogenetic role for the Hp 2-2 phenotype (3). First, the clearance of the Hb/Hp complex is Hp phenotype dependent with Hp 1-1/Hb complexes being cleared more efficiently than Hp 2-2/Hb complexes (4). Second, the Hp 2-2/Hb complex is an inferior antioxidant compared to the Hp 1-1/Hb complex in studies measuring conjugated diene formation of linolenic acid or TBARS formation by oxidized LDL (5). Third, Hp 1-1 is more efficient in preventing heme release from Hp/Hb complexes than Hp 2-2 (6), a finding that may help explain differences in antioxidant capabilities between the different Hp types. Finally, recent studies show impaired reverse cholesterol transport in diabetics carrying the Hp 2-2 genotype, presumably due to the binding of Hp 2-2/Hb complexes to HDL followed by subsequent iron-mediated oxidative damage (7).
The presence of the Hp 2-2 phenotype in diabetic individuals predicts cardiovascular risk. Several longitudinal studies have established that the Hp 2-2 phenotype is an independent risk factor for cardiovascular disease in type 1 and in type 2 diabetics (3, 8-11). Although the distribution of Hp phenotypes is not different in individuals with or without diabetes (1), the Hp 2-2 phenotype was shown to be a risk factor only in patients with diabetes. This may occur because in a diabetic patient, glycosylation of hemoglobin and the reduction of macrophages expressing the CD163 receptor may contribute to the increase in oxidative stress and tissue damage (4, 12). It has been shown that the oxidation of LDL by glycosylated hemoglobin is not completely blocked by binding to Hp (4) and the impaired removal of the complexes results in their localization in HDL particles (13). This increased oxidation of lipoproteins by the Hp 2-2/Hb complexes likely contributes to the development of vascular complications in diabetics.

Principle of the Test

- Haptoglobin (Hp) Typing ELISA plates are supplied coated with purified monoclonal antibody (mAb) directed against Hp.
- The serum to be tested is diluted and incubated in the Hp ELISA plate. In this step Hp in the serum binds to the immobilized antibody.
- Non-bound Hp is removed by washing.
- Horseradish Peroxidase (HRP) conjugated monoclonal antibody to Hp is added. Note: the same mAb in the unconjugated form was used to coat the microtiter wells. Since Hp 1-1 is dimeric, at most only one HRP conjugated mAb can bind per dimer attached to the well. However, Hp 2-1 and Hp 2-2 are polymeric and can potentially bind 2-8 HRP conjugated mAbs.
- Unbound conjugate is removed by washing.
- TMB substrate reagent is added resulting in the development of a blue color.

- The blue color development is stopped with the addition of stop solution changing the color to yellow.
- Absorbance is measured using a spectrophotometer at 450 nm.
- The absorbance of each sample is compared to a user-calculated cut-off to determine its Hp phenotype.

Assay Procedure

Obtain appropriate number of microtiter wells coated with anti-Hp monoclonal antibody from foil pack.
↓
Add 100 ul Positive Controls (Hp 1-1, Hp 2-1, Hp 2-2), 100 ul Blank Control, and diluted samples.
↓
Cover plate and incubate 30 min at room temp (22-28°C) while shaking at 750 rpm.
↓
Wash 5 times with 1x wash buffer.
↓
Add 100 ul of 1x HRP conjugated anti-Hp antibody to each well.
↓
Cover plate and incubate 30 min at room temp (22-28°C) while shaking at 750 rpm.
↓
Wash 5 times with 1x wash buffer.
↓
Add 100 ul TMB substrate to each well.
↓
Cover plate and incubate 20 min at room temp (22-28°C) while shaking at 750 rpm.
↓
Stop reaction by adding 100 ul of Stop Solution to each well.
↓
Read absorbance at 450 nm in a microliter plate reader.
↓
Calculate and interpret results.

Kit contents

1. **Anti-Hp antibody-coated microtiter plate:** 96 break-apart wells (8 x 12) coated with anti haptoglobin monoclonal antibody, packed in an aluminum pouch containing a desiccant card.
   1 Plate
2. **Concentrated wash buffer (20X):** Phosphate buffered saline (PBS) containing Tween 20.
   1 bottle, 100 ml
3. **Sample diluent Hp:** A ready-to-use buffer solution. Contains less than 0.05% Proclin as a preservative.
   1 Bottle, 15 ml
4. **Conjugate diluent:** A ready to use buffer solution. Contains less than 0.05% commercial preservatives.
5. **Concentrated HRP conjugate (200x):** HRP conjugated anti-Hp antibody solution. Contains less than 0.05% Proclin as a preservative.
   1 Vial , 0.150 ml
6. **TMB substrate:** A ready-to-use solution. Contains 3, 3', 5, 5' – tetramethylbenzidine as a chromogen and peroxide as a substrate.
   1 Bottle, 14 ml
7. **Stop solution:** A ready-to-use 1 M H_2SO_4 solution (1 bottle).
   1 Bottle, 15 ml
8. **Positive control Hp 1-1:** A ready-to-use solution containing Hp 1-1 positive human serum. Contains less than 0.05% Proclin as a preservative.
   1 Vial, 2 ml
9. **Positive control Hp 2-1:** A ready-to-use solution containing Hp 2-1 positive human serum. Contains less than 0.05% Proclin as a preservative.
   1 Vial, 2 ml
10. **Positive control Hp 2-2:** A ready-to-use solution containing Hp 2-2 positive human serum. Contains less than 0.05% Proclin as a preservative.
    1 Vial, 2 ml
11. **Plate sealing film**
12. **Instruction manual**

Materials Required But Not Supplied

1. Clean test tubes for dilution of patients' sera.
2. Adjustable micropipettes (5-50, 50-200, 200-1000 ul) and or multichannel pipettes and disposable tips.
3. Microplate shaker capable of shaking microplates at 750 rpm.
4. Microplate washer.
5. Absorbent paper.
6. ELISA reader with a 450 nm filter.
7. Distilled or double deionized water.

Warning and Precautions

1. ***CAUTION:*** This kit contains human serum from individuals who tested negative for HbsAg, HIV 1 and 2, and HCV by FDA approved methods. However, no method can completely assure absence of these agents. Therefore, all human blood products, including serum and plasma samples, should be considered as potentially infectious. Handling should be as defined by an appropriate biohazard safety guideline or regulation, where it exists (15).

2. Avoid contact with 1 M H_2SO_4. It may cause skin irritation and burns. If contact occurs, wash with copious amounts of water and seek medical attention if irritation persists.
3. Do not use reagents after expiration date and do not mix or use components from kits with different lot numbers.
4. TMB-Substrate solution is an irritant material to skin and mucous membranes.

Storage and Shelf Life of Reagents

1. Store the unopened kit at 2-8°C upon receipt and when not in use, until expiration shown on the kit label. Refer to the package label for expiration date. Exposure of the originally stoppered or sealed components to ambient temperature for a few hours will not cause damage to the reagents. **DO NOT FREEZE!**
2. Once the kit is opened, its shelf life is 90 days.
3. Unused strips must be resealed in the aluminum pouch with the desiccant card by closing the zip lock closure.
4. Crystals may form in the 20X concentrated wash buffer during cold storage. Re-dissolve the crystals by warming the buffer to 37°C before diluting. Once diluted, the solution may be stored at 2-8°C up to twenty-one days.

Specimen Collection and Preparation

1. Whole blood should be collected using standard venipuncture techniques. Serum or plasma should be stored at 2-8°C for up to 48 hours. Store at -20°C or below for long term storage.
2. Avoid grossly hemolytic (bright red) samples (after centrifugation). Hemolyzed samples may give inaccurate results (see acceptable levels of endogenous interfering substances).
3. Specimens should not be repeatedly frozen and thawed prior to testing. DO NOT store in "frost free" freezers, which may cause occasional thawing.

Test Procedure

A. Preparation of Reagents.

1. All reagents should be allowed to reach room temperature (22-28-°C). Mix well the ready-to use positive controls (Hp 1-1, Hp 2-1, and Hp 2-2).
2. Determine the total number of samples to be tested. In addition to the unknown samples, the following must be included in each test: one blank sample and three positive control samples.
3. Withdraw the microtiter plate from its aluminum pouch by cutting one end near the seal. Leave the required number of strips (according to the total number of samples to be tested) in the 96-well frame. Place the remaining strips back in the aluminum pouch with the desiccant card and seal with zip lock closure.
4. Prepare working wash buffer by adding 50 ml 20X wash buffer stock to 950 ml deionized water. Mix well.

B. Incubation of sera samples and controls

5. Dilute unknown serum samples 1:10 with sample dilution buffer. For example, add 15 ul patient serum to 135 ul sample dilution buffer in a separate eppendorf tube. Mix gently.
6. Dispense 100 ul of blank (sample diluent), positive controls, and diluted serum samples into separate wells of the test strip(s).
7. Incubate for 30 min at room temperature (22-28°C) on a plate shaker at 750 rpm.
8. Discard the liquid content of the wells.
9. Washing step: Fill each well with wash buffer (300-350 ul) up to the end of the well and discard the liquid. Strike the wells onto absorbent paper to remove residual buffer droplets. Repeat this step four times, for a total of five washing steps.

C. Incubation with conjugate

10. Dilute concentrated HRP conjugate 1:200 with conjugate diluent.
11. Dispense 100 ul diluted conjugate to each well.
12. Incubate for 30 min at room temperature (22-28°C) on a plate shaker at 750 rpm.
13. Discard the liquid content of the wells and wash as described in step 9.

D. Incubation with TMB substrate

14. Dispense 100 ul TMB substrate into each well.
15. Incubate for 20 min at room temperature (22-28°C) on a plate shaker at 750 rpm.
16. Stop reaction by adding 100 ul stop solution to each well.

Determination of Results

A. Determination of Absorbance

Determine the absorbance at 450 nm and record results. Determination should not exceed 30 minutes following stopping of chromogenic reaction.

Note: any air bubbles should be removed before reading. The bottom of the ELISA plate should be carefully wiped.

B. Cut-off Calculation

A calculated cutoff must be determined by multiplying the O.D. obtained for Hp 2-2 by 0.6.

Test Validation

The following criteria must be met for the test to be valid. If these criteria are not met, the test should be considered invalid and should be repeated.

1. O.D. of the blank should be less than 0.2.
2. O.D. of the Hp 1-1 positive control should be less than 0.2.
3. O.D. of the Hp 2-1 positive control should be between 0.2 and the calculated cut-off (value of the O.D. obtained for the Hp 2-2 positive control multiplied by 0.6).

Interpretation of Results

OD range	Hp type in sample
Less than 0.2	**Hp 1-1**
Between 0.2 and calculated cutoff	**Hp 2-1**
Greater than calculated cutoff	**Hp 2-2**

Acceptable levels of endogenous interfering substances

Endogenous interfering substance	Acceptable level
Hemoglobin	2 g/L
Ascorbic acid (vitamin C)	170 µM
Aspirin	3.62 mM

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Limitations of the Procedure

4. The results obtained from the use of this kit should be used as an adjunct to other diagnostic procedures and information available to the physician.
5. Patient samples may contain human anti-mouse antibodies (HAMA) that are capable of giving falsely elevated or depressed results with assays that utilize mouse monoclonal antibodies. This assay has been designed to minimize interference from HAMA-containing specimens. Never the less, complete elimination of this interference from all patient specimens cannot be guaranteed.

Performance Characteristics

	Hp 1-1	Hp 2-1	Hp 2-2
Sensitivity	97.8% 90/92	98.3% 115/117	100.0% 108/108
Specificity	99.1% 223/225	99.5 199/200	99.5% 208/209
Agreement*	97.8% 90/92	99.1 115/116	99.1% 108/109

* *With standard electrophoretic procedures (16).*

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