

Rabbit Haptoglobin ELISA Quantitation Kit

Manual

Immunoperoxidase Assay for Determination of Haptoglobin in Rabbit Sera.
This kit is for research use only, and is not for use in
diagnostic procedures.

INTENDED USE

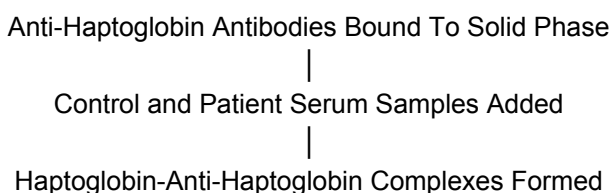
The HAPTOGLOBIN test kits are a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring HAPTOGLOBIN in serum or plasma of rabbits.

INTRODUCTION

Haptoglobin (TX) is a metal-combining protein that reversibly binds to acid-soluble iron in plasma. It functions to transport iron to the bone marrow and to tissue storage organs such as the liver. Haptoglobin also participate in the regulation and control of iron absorption and protects against iron intoxication. Like haptoglobin, the carrier of hemoglobin, Haptoglobin is synthesized in the liver, but unlike haptoglobin Haptoglobin is returned to the circulation after unloading its iron in the reticuloendothelial system. This ELISA kit can be used to measure Haptoglobin in serum, tissue extracts and other biological fluids.

PRINCIPLE OF THE TEST

The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay the HAPTOGLOBIN present in serum sample reacts with the anti-HAPTOGLOBIN antibodies which have been adsorbed to the surface of polystyrene microtitre wells. After the removal of unbound serum proteins by washing, anti-HAPTOGLOBIN antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes with the previously bound serum HP. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of HAPTOGLOBIN in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of HAPTOGLOBIN in the test sample. The quantity of HAPTOGLOBIN in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for serum dilution.



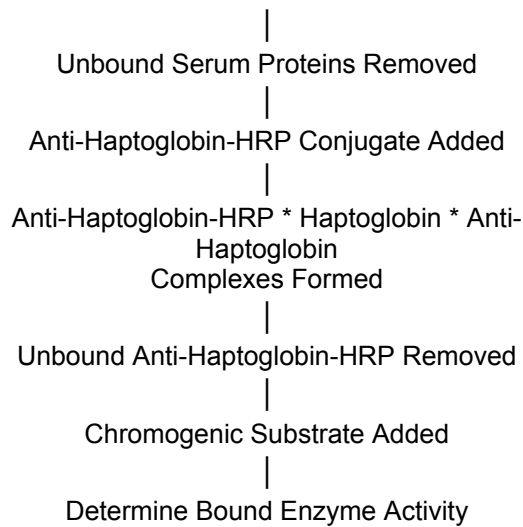


FIGURE 1.

REAGENTS

(Quantities sufficient for 96 determinations)

1. DILUENT CONCENTRATE

One bottle containing 50 mL of a 5X concentrated phosphate buffered saline (PBS) solution containing bovine serum albumin, 0.25% Tween, and 0.1% Proclin 300 as a preservative.

2. WASH SOLUTION CONCENTRATE

One bottle containing 50 mL of a 20X concentrated phosphate buffered saline (PBS) solution containing 0.5% Tween.

3. ENZYME-ANTIBODY CONJUGATE 100X

One vial containing 200 μ L of affinity purified anti-Pig HAPTOGLOBIN antibody conjugated with horseradish peroxidase in a stabilizing buffer.

4. CHROMOGEN-SUBSTRATE SOLUTION

One vial containing 12 mL of 3,3',5,5'-tetramethybenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3.

5. STOP SOLUTION

One vial containing 12 mL 0.3 M sulfuric acid.

WARNING: Avoid contact with skin.

6. ANTI-RABBIT HAPTOGLOBIN ELISA MICRO PLATE

Twelve removable eight (8) well micro well strips in well holder frame. Each well is coated with affinity purified anti-Rabbit Haptoglobin.

7. RABBIT HAPTOGLOBIN STANDARDS

One vial containing 1.4 mL of Purified Rabbit Haptoglobin (RA-15HP) at 400 ng/mL.

FOR IN VITRO USE ONLY

REAGENT PREPARATION

1. DILUENT CONCENTRATE

The Diluent Solution supplied is a 5X Concentrate and must be diluted 1:5 with distilled or deionized water.

2. WASH SOLUTION CONCENTRATE

The Wash Solution supplied is a 20X Concentrate and must be diluted 1:20 with distilled or deionized water. Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.

3. ENZYME-ANTIBODY CONJUGATE

The required amount of working conjugate solution for each microtitre plate is prepared by adding 100 µL Enzyme-Antibody Conjugate to 10 mL of Diluent. Mix uniformly, but gently. Avoid foaming.

4. CHROMOGEN-SUBSTRATE SOLUTION

Ready to use as supplied.

5. STOP SOLUTION

Ready to use as supplied.

6. ANTI-RABBIT HAPTOGLOBIN ELISA MICRO PLATE

Ready to use as supplied.

7. RABBIT HAPTOGLOBIN STANDARDS

Purified Rabbit Haptoglobin needs to be diluted according to the chart below for each run. Mix well between each step. Avoid foaming. For samples containing very low levels of HP, it is possible to extend the utility of the lower detection limit of this assay by further serial 2-fold dilution of standard # 5.

Standard	ng/mL	Volume added to 1x Diluent	Volume of 1x Diluent
1	200	0.3 ml Purified Rabbit HRP Calibrator	0.3 ml
2	100	0.3 ml standard 1	0.3 ml
3	50	0.3 ml standard 2	0.3 ml
4	25	0.3 ml standard 3	0.3 ml
5	12.5	0.3 ml standard 4	0.3 ml

STORAGE AND STABILITY

The expiration date for the package is stated on the box label.

1. DILUENT

The 5X Diluent Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4-8°C.

2. WASH SOLUTION

The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions can be stored at room temperature (16-25°C) or at 4-8°C.

3. ENZYME-ANTIBODY CONJUGATE

Undiluted horseradish peroxidase anti-HX conjugate should be stored at 4-8°C and diluted immediately prior to use. The working conjugate solution is stable for one day.

4. CHROMOGEN-SUBSTRATE SOLUTION

The Substrate Solution should be stored at 4-8°C and is stable until the expiration date.

5. STOP SOLUTION

The Stop Solution should be stored at 4-8°C and is stable until the expiration date.

6. ANTI-RABBIT HAPTOGLOBIN ELISA MICRO PLATE

Anti-Rabbit HP coated wells are stable until the expiration date listed on the package label, and should be stored at 4-8°C.

7. RABBIT HAPTOGLOBIN STANDARDS

Undiluted Purified Rabbit Haptoglobin should be stored FROZEN for storage longer than 7 days until the expiration date. For storage less 7 days can be at 4C and diluted immediately before use. Avoid multiple freeze-thaw cycles. The working standard solution is stable for one day.

INDICATIONS OF INSTABILITY

If the test is performing correctly, the results observed with the standard solutions should be within 20 % of the expected values.

SPECIMEN COLLECTION AND PREPARATION

Blood should be collected by venipuncture and the serum separated from the cells, after clot formation, 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-thaw cycles.

1. Precautions

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds.

2. Additives and Preservatives

No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

3. Known interfering substances

Azide and thimerosal at concentrations higher than 0.1% inhibits the enzyme reaction.

MATERIAL PROVIDED

See "REAGENTS"

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipette (2 μ L to 200 μ L) for making and dispensing dilutions
- Test tubes
- Microtitre washer/aspirator
- Distilled or Deionized H₂O
- Microtitre Plate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Vortex mixer

ASSAY PROCEDURE

DILUTION OF SERUM SAMPLES

The assay for quantification of Haptoglobin in serum requires that each test sample be diluted before use. A 1:5,000 dilution is appropriate for most serum or plasma samples. For absolute quantification, samples that yield results outside the range of the standard curve, a lesser or greater dilution might be required.

1. To prepare a 1:5,000 dilution of sample, transfer 5 μ L of sample to 495 μ L of diluent. This gives you a 1:100 dilution. Next, dilute the 1:100 samples by Haptoglobing 10 μ L, to 490 μ L of diluent. You now have a 1:5,000 dilution of your sample. Mix thoroughly at each stage.

PROCEDURE

Bring all reagents to room temperature before use.

1. Add 100 μ L of Diluent to each of the wells in 1A & 2A. These will serve for an evaluation of the background associated with the assay.

2. Pipette 100 μ L of
Standard 1 (200 ng/ml) into wells 1B & 2B
Standard 2 (100 ng/ml) into wells 1C & 2C
Standard 3 (50 ng/ml) into wells 1D & 2D
Standard 4 (25 ng/ml) into wells 1E & 2E
Standard 5 (12.5 ng/ml) into wells 1F & 2F

3. Pipette 100 μ L of serum sample (test sample 1) into wells 1G & 2G. The next sample goes in wells 1H & 2H, the next in 3A & 4A and so on.

4. Incubate the micro titer plate at 22°C (room temperature) for sixty (60 \pm 2) minutes. Keep plate level during incubation.

5. Following incubation, aspirate the contents of the wells.

6. Completely fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. If washing manually; completely fill wells with wash buffer, invert the plate and pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of four washes.

7. Pipette 100 μ L of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at 22°C (room temperature) for fifteen (15 ± 2) minutes.
8. Wash and blot the wells as described in Step 6.
9. Pipette 100 μ L of TMB Substrate Solution into each well.
10. Incubate at room temperature for precisely ten (10) minutes.
11. After ten (10) minutes, add 100 μ L of Stop Solution to each well.
12. Determine the absorbance (450 nm) of the contents of each well. Calibrate the plate reader to air.

STABILITY OF THE FINAL REACTION MIXTURE

The absorbance of the final reaction mixture can be measured up to 2 hours after the addition of the Stop Solution. However, good laboratory practice dictates that the measurement be made as soon as possible.

RESULTS

1. Subtract the average background value from the test values for each sample.
2. Using the results observed for the standards construct a Standard Curve. The appropriate curve fit is that of a four-parameter logistics curve. A second order polynomial (quadratic) or other curve fits may also be used.
3. Interpolate test sample values from standard curve. Correct for sera dilution factor to arrive at Haptoglobin concentration in original sample.

QUALITY CONTROL

In accord with good laboratory practice, the Assays for specific HP require meticulous quality control. Each laboratory should use routine quality control procedures to establish inter- and intra-assay precision and performance characteristics.

LIMITATION OF THE PROCEDURE

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the information contained in the package insert instructions and with adherence to good laboratory practice.
2. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or deionized water, and accuracy of reagent and sample pipettings.

REFERENCES

1. Tillett, W.S. and T. Francis. 1930. Serological reactions in pneumonia with non-protein somatic fraction of pneumococcus. J. Exp Med. 52:561-571.
2. Eckersal, P.D. 2000. Recent advances and future prospects for the use of acute phase proteins and markers of disease in animals. Revue Med. Vet. 151(7): 577-584.

TROUBLESHOOTING

The following are some common problems encountered with the use of ELISA kits, and some of the causes of these problems.

1. **Problem: Low absorbance**
 - ☐ Incorrect dilutions or pipetting errors.
 - ☐ Improper incubation times
 - ☐ Improper mixing of the TMB substrate. Each component is mixed in equal parts.
 - ☐ Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB, 490 nm for OPD, or 405 nm for ABTS.
 - ☐ Kit materials or reagents are contaminated or expired.
 - ☐ Incorrect reagents used.
2. **Problem: High Absorbance**
 - ☐ Cross contamination from other samples or positive control.
 - ☐ Incorrect dilutions or pipetting errors.
 - ☐ Improper washing.
 - ☐ Wrong filter on microtiter reader.
 - ☐ Contaminated buffers or enzyme substrate.
 - ☐ Improper incubation times.
 - ☐ Kit materials or reagents are contaminated or expired.
3. **Problem: Poor Duplicates**
 - ☐ Poor mixing of specimens.
 - ☐ Incorrect dilutions or pipetting errors.
 - ☐ Technical error.
 - ☐ Inconsistency in following ELISA protocol.
 - ☐ Inefficient washing.
4. **Problem: All wells are positive**
 - ☐ Contaminated buffers or enzyme substrate.
 - ☐ Incorrect dilutions or pipetting errors.
 - ☐ Kit materials or reagents are contaminated or expired.
 - ☐ Inefficient washing.
5. **Problem: All wells are negative**
 - ☐ Procedure not followed correctly.
 - ☐ Contaminated buffers or enzyme substrate.
 - ☐ Contaminated conjugate.
 - ☐ Kit materials or reagents are contaminated or expired.

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