AssayLite™

Human Ceruloplasmin and Haptoglobin Multiplex Fluorescent Immunoassay Kit

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This product is manufactured under patent pending technology by Assaypro LLC

For any questions regarding troubleshooting or performing the assay, please contact our support team at support@assaypro.com.

Table of Contents

Assay Summary

Step 1. Add 50 µl of Standard/Sample and 50 µl of Fluorescent Probe per well.
Incubate 2 hours at 37°C.

Step 2. Wash, then add 50 µl of Stabilizing Solution per well.

Step 3. Human Ceruloplasmin: Read at EX 485/20 nm, EM 575/15 nm; Human Haptoglobin: Read at EX590/10 nm, EM660/10 nm

Symbol Key

Consult instructions for use.

Assay Mechanism

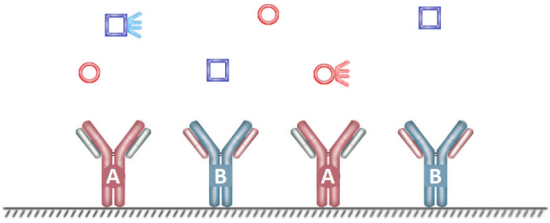


Figure 1. Two different types of antibodies are coated uniformly on the microplate. Standard or Sample and the Fluorescent Probe are added to each well.

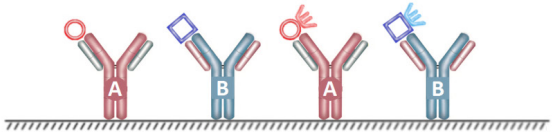
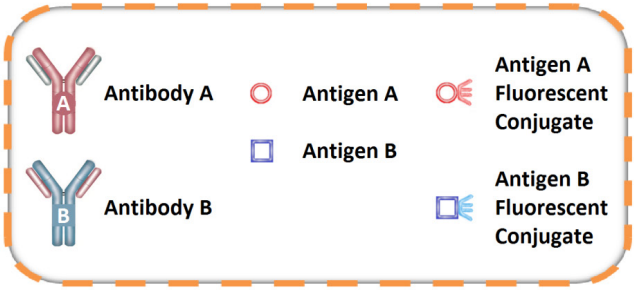


Figure 2. Fluorescent Probe competes with the non-labeled protein for binding to the antibody. The blank (Diluent only, 0 µg/ml) shows the highest fluorescent signal. Signal decreases when the non-labeled protein concentration increases.



Assay Template

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Human Ceruloplasmin and Haptoglobin Multiplex Fluorescent Immunoassay Kit

Catalog No. FH100311M2

Sample insert for reference use only

Introduction

Ceruloplasmin is an abundant alpha-2-serum glycoprotein that contains 95% of the copper found in the plasma of vertebrate species (1). Ceruloplasmin is a copper-binding protein that normally removes iron from cells by its ferroxidase activity. Low levels of ceruloplasmin lead to the abnormal deposition of iron in cells, including those of the pancreas, liver, retina, and the basal ganglia region of the brain. Some diseases associated with ceruloplasmin are Wilson's disease (2), Hemochromatosis (3), Menkes disease, (4) and Aceroluplasminemia (1).

Haptoglobulin (Hpt) is a plasma protein with hemoglobin-binding capacity and plasma glycoproteins that form a stable complex with hemoglobin to aid the recycling of heme iron. It is a well-known marker of hemolysis (5). High haptoglobulin level in plasma was associated with an increased cardiovascular risk in obese men (6), inflammation (7), atherosclerosis (8), and systemic sclerosis (9).

Principle of the Assay

The AssayLite™ Human Ceruloplasmin and Haptoglobin Multiplex Multiplex Fluorescent Immunoassay Kit employs a **quantitative competitive fluorescent probe technique** that measures Ceruloplasmin and Haptoglobin in human **plasma, serum, saliva, milk, and cell culture samples** in approximately 2 hours. A polyclonal antibody specific for human Ceruloplasmin and a polyclonal antibody specific for human Haptoglobin have been pre-coated onto a 96-well opaque polystyrene microplate with removable strips. Human Ceruloplasmin and human Haptoglobin standards and samples are competed with a human Ceruloplasmin and human Haptoglobin fluorescent probe. All unbound material is washed away before the endpoint fluorescence is measured.

Caution and Warning

- This product is for **Research Use Only** and is not intended for use in diagnostic procedures.
- Prepare all reagents (diluent buffer, wash buffer, standard, and fluorescent probes) 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 insert. However, the user should determine the optimal dilution factor.
- The kit should not be used beyond the expiration date.
- Avoid direct light exposure to the assay.
- Store fluorescent probes in a dark place. Do not freeze.

Reagents

- **Human Ceruloplasmin and Haptoglobin Microplate:** A 96-well opaque polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human Ceruloplasmin and a polyclonal antibody against Haptoglobin.
- **Sealing Films:** Each kit contains 3 precut, pressure sensitive aluminum sealing films that can be cut to fit the format of the individual assay.
- **Human Ceruloplasmin and Haptoglobin Standard:** Human Ceruloplasmin and Haptoglobin in a buffered protein base (Human Ceruloplasmin: 400 µg, Human Haptoglobin: 240 µg, lyophilized).
- **Red Fluorescent Human Ceruloplasmin Probe (16x):** 1 vial, lyophilized.
- **Blue Fluorescent Human Haptoglobin Probe (6x):** 3 vials, lyophilized.
- **EIA Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml, 1 bottle).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml, 1 bottle).
- **Stabilizing Solution (1x):** A solution to stabilize the fluorescent component (8 ml, 1 bottle).

Storage Conditions

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store Microplate, Diluent Concentrate (10x), Stabilizing Solution, and Wash Buffer at 2-8°C.
- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.
- Store Standard at 2-8°C before reconstituting with Diluent and at -20°C after reconstituting with Diluent.
- Store Fluorescent Probes in a dark place at 2-8°C. Do not freeze.

Other Supplies Required

- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl, and multiple channel)
- Deionized or distilled reagent grade water
- Incubator (37°C)
- Fluorescent Microplate Reader

Fluorescent Microplate Reader (BioTek® Synergy H1F, filter-based reader; available for order from Assaypro)

Sample Collection, Preparation, and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x *g* for 10 minutes and collect plasma. A 1600-fold sample dilution is suggested into EIA Diluent *(suggested dilution factor only; user should determine optimal dilution factor depending on application needs).* The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x *g* for 10 minutes and remove serum. A 1600-fold sample dilution is suggested into EIA Diluent *(suggested dilution factor only; user should determine optimal dilution factor depending on application needs).* The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Milk:** Collect milk using sample tube. Centrifuge samples at 800 x *g* for 10 minutes. A 40-fold sample dilution is suggested into EIA Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Saliva:** Collect saliva using sample tube. Centrifuge samples at 800 x *g* for 10 minutes. A 20-fold sample dilution is suggested into EIA Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatant:** Centrifuge cell culture media at 1500 rpm for 10 minutes at 4°C to remove debris and collect supernatant. Samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.
- **Cell Lysate:** Rinse cell with cold PBS and then scrape the cell into a tube with 5 ml of cold PBS and 0.5 M EDTA. Centrifuge suspension at 1500 rpm for 10 minutes at 4°C and aspirate supernatant. Resuspend pellet in ice-cold Lysis Buffer (10 mM Tris, pH 8.0, 130 mM NaCl, 1% Triton X-100, protease inhibitor cocktail). For every 1 x 10^6 cells, add approximately 100 µl of ice-cold Lysis Buffer. Incubate on ice for 60 minutes.

Centrifuge at 13000 rpm for 30 minutes at 4°C and collect supernatant. Samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.

Applicable samples may also include biofluids, cell culture, and tissue homogenates. If necessary, user should determine optimal dilution factor depending on application needs.

Refer to Dilution Guidelines for further instruction.

Guidelines for Dilutions of 100-fold or Greater (*for reference only; please follow the insert for specific dilution suggested)*	
100x	**10000x**
A) 4 µl sample: 396 µl buffer (100x) = 100-fold dilution *Assuming the needed volume is less than or equal to 400 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) = 10000-fold dilution *Assuming the needed volume is less than or equal to 400 µl.*
1000x	**100000x**
A) 4 µl sample : 396 µl buffer (100x) B) 24 µl of A : 216 µl buffer (10x) = 1000-fold dilution *Assuming the needed volume is less than or equal to 240 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) C) 24 µl of B : 216 µl buffer (10x) = 100000-fold dilution *Assuming the needed volume is less than or equal to 240 µl.*

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the EIA Diluent Concentrate 10-fold with reagent grade water to produce a 1x solution. Store for up to 30 days at 2-8°C.
- **Human Ceruloplasmin and Haptoglobin Standard:** Reconstitute the Human Ceruloplasmin and Haptoglobin Standard (Human Ceruloplasmin: 80 µg, Human Haptoglobin: 240 µg) with 1 ml of EIA Diluent to generate a standard stock solution (Human Ceruloplasmin: 80 µg/ml, Human Haptoglobin: 240 µg/ml). Allow the vial to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting from the standard stock solution 4-fold with EIA Diluent to produce a standard curve that measures both Human Ceruloplasmin and Human Haptoglobin proteins simultaneously. EIA Diluent serves as the zero standard (0 µg/ml). Any remaining stock solution should be stored at -20°C and used within 30 days. Avoid repeated freeze-thaw cycles.

Standard Point	Dilution	[Human Ceruloplasmin] (µg/ml)	[Human Haptoglobin] (µg/ml)
P1	1 part stock + 3 parts EIA Diluent	20	
P2	1 part P1 + 3 parts EIA Diluent	5	15
P3	1 part P2 + 3 parts EIA Diluent	1.25	3.75
P4	1 part P3 + 3 parts EIA Diluent	0.313	0.94
P5	1 part P4 + 3 parts EIA Diluent	0.078	0.23
P6	1 part P5 + 3 parts EIA Diluent	0.0195	0.059
P7	1 part P6 + 3 parts EIA Diluent		0.015
P8	EIA Diluent	0.0	0.0

- **Red Fluorescent Human Ceruloplasmin Probe (16x):** Reconstitute the fluorescent probe with 1 ml of EIA Diluent to produce a 16 x stock solution. Allow the probe to sit for 10 minutes with gentle agitation prior to use. Any remaining stock solution should be stored at 2-8°C and used within 10 days. Do not freeze.
- **Blue Fluorescent Human Haptoglobin Probe (6x):** Reconstitute the fluorescent probe with 1 ml of EIA Diluent to produce a 6 x stock. Allow the probe to sit for 10 minutes with gentle agitation prior to use. Any remaining stock solution should be stored at 2-8°C and used within 10 days. Do not freeze.

    - After Red and Blue Fluorescent Probes are reconstituted and ready, combine each probes to the desired volume to generate the **Human Ceruloplasmin and Haptoglobin Fluorescent Probe Mixture**.

      Example:
      Combine the following reagents to generate working solution to run full 96 well plate (total volume is 7000 ul).

        1. 3792 ul of 1x EIA Diluent
        2. 875 µl Red Fluorescent Human Ceruloplasmin Probe
        3. 2333 µl Blue Fluorescent Human Haptoglobin Probe

- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

Dilute the Wash Buffer Concentrate 20-fold with reagent grade water to produce a 1x solution.

Assay Procedure

- Prepare all reagents, standard solution, and samples as instructed. Bring all reagents to room temperature before use. The assay is incubated at 37°C.
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Human Ceruloplasmin and Haptoglobin Standard or sample to each well, and immediately add 50 µl of Human Ceruloplasmin and Haptoglobin Fluorescent Probe Mixture to each well (on top of the standard or sample). Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed. Cover wells with a sealing film and incubate for 2 hours at 37°C. Start the timer after the last addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a machine, wash six times with 300 µl of Wash Buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.
- Immediately add 50 µl of Stabilizing Solution to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed.
- **Human Ceruloplasmin:** Read the endpoint fluorescence on a microplate reader at an excitation wavelength of 485/20 nm and emission wavelength of 575/15 nm.
- **Human Haptoglobin:** Read the endpoint fluorescence on a microplate reader at an excitation wavelength of 590/10 nm and emission wavelength of 660/10 nm.
- **Human Ceruloplasmin:** For the Synergy H1F, a gain of 75 is suggested; however, the user should determine the optional gain/amplification.
- **Human Haptoglobin:** For the Synergy H1F, a gain of 90 is suggested; however, the user should determine the optional gain/amplification.

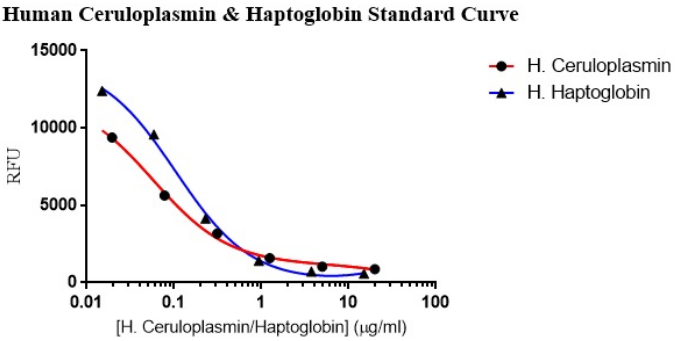
Data Analysis

- 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 emitted fluorescence 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.



Note: Standard curve reading with Biotek® Synergy H1F

Performance Characteristics

Reader	Standard Point	µg/ml	Human Ceruloplasmin Average Red RFU
BioTek – Synergy H1F	P1	20	868
	P2	5	1036
	P3	1.25	1577
	P4	0.313	3164
	P5	0.078	5629
	P6	0.0195	9385
	P7	0.0	11278

Reader	Standard Point	µg/ml	Human Haptoglobin Average Blue RFU
BioTek – Synergy H1F	P1	15	577
	P2	3.75	715

	P3	0.94	1400
	P4	0.23	4144
	P5	0.059	9575
	P6	0.015	12397
	P7	0.0	13135

- The minimum detectable dose of human Ceruloplasmin as calculated by 2SD from the mean of a zero standard was established to be 0.01 µg/ml.
- The minimum detectable dose of Human Haptoglobin as calculated by 2SD from the mean of a zero standard was established to be 0.01 µg/ml.
- Intra-assay and inter-assay coefficients of variation were 4.3 % and 8.8% respectively.

Reference Value

- Normal human Ceruloplasmin plasma levels range from 0.2 – 0.6 mg/ml.
- Plasma and serum samples from healthy adults were tested (n=20). On average, human Ceruloplasmin level was 300 µg/ml.
- The normal human plasma levels of haptoglobin are 0.3 – 2 mg/ml.
- Human plasma and serum samples from healthy adults were tested (n=40). On average, haptoglobin level was 902 µg/ml.

Validation:

- The kit has been validated against Human Ceruloplasmin ELISA Kit and Human Haptoglobin ELISA Kit.

Specification

Probe Used	**H. Ceruloplasmin (Purity > 95%)**	**Human Haptoglobin (Purity > 95%)**
Red Fluorescent Human Ceruloplasmin Probe	90 - 110%	0.4 - 2.6%
Blue Fluorescent Human Haptoglobin Probe	0.8 - 7.5%	91 - 108%

Recovery

Name	**Human Ceruloplasmin**	**Human Haptoglobin**
Standard Added Value	0.313 – 5 µg/ml	0.23 - 3.75 µg/ml
Recovery %	96 – 104%	92 – 105%
Average Recovery %	99%	98%

Linearity

Average Percentage of Expected Value (%)		
Sample Dilution for Plasma	Human Ceruloplasmin	Human Haptoglobin
800x	103%	102%
1600x	97%	98%
3200x	90%	93%

Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of expired components	• Check the expiration date listed before use. • Do not interchange components from different lots.
	Improper wash step	• Check that the correct wash buffer is being used. • Check that all wells are empty after aspiration. • Check that the microplate washer is dispensing properly. • If washing by pipette, check for proper pipetting technique.
	Splashing of reagents while loading wells	• Pipette properly in a controlled and careful manner.
	Inconsistent volumes loaded into wells	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the Standard and Fluorescent Probe after reconstitution. • Thoroughly mix dilutions.
	Improperly sealed microplate	• Check the microplate pouch for proper sealing. • Check that the microplate pouch has no punctures. • Check that three desiccants are inside the microplate pouch prior to sealing.
Unexpectedly Low or High Signal Intensity	Microplate was left unattended between steps	• Each step of the procedure should be performed uninterrupted.
	Omission of step	• Consult the provided procedure for complete list of steps.
	Steps performed in incorrect order	• Consult the provided procedure for the correct order.
	Insufficient amount of reagents added to wells	• Check pipette calibration. • Check pipette for proper performance.
	Wash step was skipped	• Consult the provided procedure for all wash steps.
	Improper wash buffer	• Check that the correct wash buffer is being used.
	Improper reagent preparation	• Consult reagent preparation section for the correct dilutions of all reagents.
	Insufficient or prolonged incubation periods	• Consult the provided procedure for correct incubation time.
	Prolonged exposure of assay or Fluorescent Probe to light	• Overexposure can affect the stability of the Fluorescent Probe, store in a dark location. • Cover and cap all reagents when not in use. • Cover assay with aluminum sealing film immediately after loading.

	Contamination of reagents	• A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporated	• Verify that the aluminum sealing film is firmly in place before placing the assay in the incubator.
	Used filters with an overlapping bandpass	• As an example, do not use a filter combination of 620/20 EX and 660/40 EM, use a 660/20 filter instead.
Deficient Standard Curve Fit	Improper pipetting	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the Standard and Fluorescent Probe after reconstitution. • Thoroughly mix dilutions.

References

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(9) Kucharz EJ *et al.* (2000) *Clin Rheumatol* 19(2): 165-6

Version 1.0

Fluorescent Microplate Reader

- BioTek® Synergy H1F, Filter-Based Reader
- Gen5 Software included
- Includes three filters designed specifically for AssayLite® Multiplex Assays. Additional filters are available for purchase
- Available for order from Assaypro for $19,950