



AssayMax™
Human Protein S-C4BP Complex
ELISA Kit

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

Thank you for choosing Assaypro.

Assay Summary

Step 1. Add 50 μ l of Standard or Sample per well.
Incubate 2 hours.

Step 2. Wash, then add 50 μ l of Biotinylated Antibody per well.
Incubate 1 hour.

Step 3. Wash, then add 50 μ l of SP Conjugate per well.
Incubate 30 minutes.

Step 4. Wash, then add 50 μ l of Chromogen Substrate per well.
Incubate 12 minutes.

Step 5. Add 50 μ l of Stop Solution per well.
Read at 450 nm immediately.

Symbol Key



Consult instructions for use.

AssayMax™ Human Protein S-C4BP Complex ELISA Kit

Catalog No. EP7758-1

Sample insert for reference use only

Introduction

Protein S, a blood coagulation inhibitor, forms a 1:1 molar complex with complement C4b-binding protein (C4BP) in human plasma with high affinity. Protein S is a single-chain vitamin K-dependent plasma glycoprotein consisting of 635 amino acids with a molecular weight of about 70 kDa. It functions as a cofactor for the anticoagulant activated protein C to inhibit blood coagulation (1-3). C4BP is a 570 kDa high molecular mass glycoprotein present in plasma in various isoforms with different alpha beta compositions. The major form of C4BP is composed of seven identical 70-kDa alpha chains containing a binding site for the complement protein C4b, and a unique 45-kDa beta chain with a binding site for protein S. In plasma, protein S is both in a free cofactor form and in a complex form with C4BP via the Gla domain on protein S (4). The complex acts as a bridge between coagulation and inflammation due to the involvement of C4BP in regulating complement activation. The complex anchors C4BP and C4b to phospholipid membranes at the site of injury to prevent inflammation. The protein S-C4BP complex binds to apoptotic cells and inhibits the phagocytosis of apoptotic cells (5-6).

Principle of the Assay

The AssayMax™ Human Protein S-C4BP Complex ELISA (Enzyme-Linked Immunosorbent Assay) Kit is designed for detection of protein S-C4BP complexes in human **plasma, serum, and urine samples**. This assay employs a quantitative **sandwich enzyme immunoassay** technique that measures human protein S-C4BP complexes in approximately 4 hours. A polyclonal antibody specific for human complement C4BP has been pre-coated onto a 96-well microplate with removable strips. Protein S-C4BP complex in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for human protein S, which is recognized by a streptavidin-peroxidase (SP) conjugate. All unbound material is washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color 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, biotinylated antibody, and SP conjugate), 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.
- Spin down the SP conjugate vial and the biotinylated antibody vial before opening and using contents.
- The Stop Solution is an acidic solution.
- The kit should not be used beyond the expiration date.

Reagents

- **Human Protein S-C4BP Complex Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human complement C4BP.
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human Protein S-C4BP Complex Standard:** Human protein S-C4BP complex in a buffered protein base (2000 pg, lyophilized).
- **Biotinylated Human Protein S-C4BP Complex Antibody (50x):** A 50-fold concentrated biotinylated polyclonal antibody against human protein S (120 µl).
- **MIX Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **SP Conjugate (100x):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate (1x):** A stabilized peroxidase chromogen substrate tetramethylbenzidine (7 ml).
- **Stop Solution (1x):** A 0.5 N hydrochloric acid solution to stop the chromogen substrate reaction (11 ml).

Storage Condition

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20°C.
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate 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.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes (1-20 μ l, 20-200 μ l, 200-1000 μ l, and multiple channel)
- Deionized or distilled reagent grade water

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 100000-fold sample dilution is suggested into MIX 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 (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 100000-fold sample dilution is suggested into MIX Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples should be aliquoted to limit repeated freeze-thaw cycles and stored at -80°C for up to 3 months. When needed, the frozen sample should be thawed rapidly in a water bath at 37°C and immediately placed on ice until use to prevent complement activation.
- **Urine:** Collect urine using sample pot. Centrifuge samples at 800 x *g* for 10 minutes. The sample is suggested for use at 1x or within the range of 2x – 10x into MIX 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.

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

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **MIX Diluent Concentrate (10x):** Dilute the MIX Diluent Concentrate 10-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved. Store for up to 30 days at 2-8°C.
- **Human Protein S-C4BP Complex Standard:** Reconstitute the Human Protein S-C4BP Complex Standard (2000 pg) with 1 ml of MIX Diluent to generate a 2000 pg/ml standard stock solution. 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 (2000 pg/ml) 2-fold with equal volume of MIX Diluent to produce 1000, 500, 250, 125, 62.5, and 31.25 pg/ml solutions. MIX Diluent serves as the zero standard (0 pg/ml). Any remaining stock solution should be stored at -20°C and used within 20 days. Avoid repeated freeze-thaw cycles.

Standard Point	Dilution	[Protein S-C4BP] (pg/ml)
P1	1 part Standard (2000 pg/ml)	2000
P2	1 part P1 + 1 part MIX Diluent	1000
P3	1 part P2 + 1 part MIX Diluent	500
P4	1 part P3 + 1 part MIX Diluent	250
P5	1 part P4 + 1 part MIX Diluent	125
P6	1 part P5 + 1 part MIX Diluent	62.5
P7	1 part P6 + 1 part MIX Diluent	31.25
P8	MIX Diluent	0.0

- **Biotinylated Human Protein S-C4BP Complex Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 50-fold with MIX Diluent to produce a 1x solution. The undiluted antibody should be stored at -20°C.
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 20-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 100-fold with MIX Diluent to produce a 1x solution. The undiluted conjugate should be stored at -20°C.

Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-25°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 Protein S-C4BP Complex Standard or sample to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last addition.
- Wash the microplate manually or automatically using a microplate washer. Invert the plate and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If washing manually, wash five times with 200 µl of Wash Buffer per well. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a microplate washer,

wash six times with 300 μ l of Wash Buffer per well; invert the plate and hit 4-5 times on absorbent material to completely remove the liquid.

- Add 50 μ l of Biotinylated Human Protein S-C4BP Complex Antibody to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 1 hour.
- Wash the microplate as described above.
- Add 50 μ l of SP Conjugate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 μ l of Chromogen Substrate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Incubate in ambient light for 12 minutes or until the optimal blue color density develops.
- Add 50 μ l of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

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 450 nm absorbance (OD) 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.

Typical Data

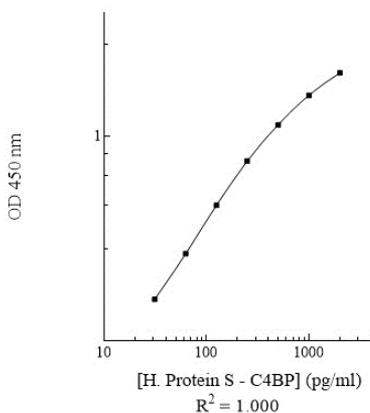
- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

Standard Point	pg/ml	OD	Average OD
P1	2000	2.042 2.018	2.030
P2	1000	1.596 1.562	1.579
P3	500	1.156 1.112	1.134
P4	250	0.748 0.758	0.753
P5	125	0.456 0.460	0.458
P6	62.5	0.254 0.278	0.266
P7	31.25	0.155 0.163	0.159
P8	0.0	0.039 0.041	0.040

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.

Human Protein S - C4BP Standard Curve



Performance Characteristics

- The minimum detectable dose of human protein S-C4BP complex as calculated by 2SD from the mean of a zero standard was established to be 7.9 pg/ml.

- Intra-assay precision was determined by testing three plasma samples twenty times in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
CV (%)	6.6%	4.3%	5.0%	11.2%	10.3%	10.5%
Average CV (%)	5.3%			10.7%		

Spiking Recovery

- Recovery was determined by spiking one plasma and one serum sample with different protein S-C4BP complex concentrations.

Sample	Unspiked Sample (pg/ml)	Spiking Value (pg/ml)	Expected	Observed	Recovery (%)
Plasma	154.131	1089.657	1243.788	1422.527	114%
		264.659	418.790	477.656	114%
		60.852	214.983	236.844	110%
Serum	207.127	1089.657	1296.784	1340.847	103%
		264.659	471.786	539.052	114%
		60.852	267.979	314.584	117%
Average Recovery (%)					112%

Linearity

- Plasma and serum samples were serially diluted to test for linearity.

Average Percentage of Expected Value (%)		
Sample Dilution	Plasma	Serum
50000x	110%	109%
100000x	102%	101%
200000x	90%	91%

Cross-Reactivity

Species	Cross-Reactivity (%)
Canine	None
Bovine	None
Equine	None
Monkey	2%
Mouse	None
Rat	None
Swine	None
Rabbit	None
Protein	Cross-Reactivity (%)
Complement C4BP	3%
Protein S	None

Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of improper components	<ul style="list-style-type: none"> Check the expiration date listed before use. Do not interchange components from different lots.
	Improper wash step	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Pipette properly in a controlled and careful manner.
	Inconsistent volumes loaded into wells	<ul style="list-style-type: none"> Pipette properly in a controlled and careful manner. Check pipette calibration. Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	<ul style="list-style-type: none"> Thoroughly agitate the lyophilized components after reconstitution. Thoroughly mix dilutions.
	Improperly sealed microplate	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Each step of the procedure should be performed uninterrupted.
	Omission of step	<ul style="list-style-type: none"> Consult the provided procedure for complete list of steps.
	Steps performed in incorrect order	<ul style="list-style-type: none"> Consult the provided procedure for the correct order.
	Insufficient amount of reagents added to wells	<ul style="list-style-type: none"> Check pipette calibration. Check pipette for proper performance.
	Wash step was skipped	<ul style="list-style-type: none"> Consult the provided procedure for all wash steps.
	Improper wash buffer	<ul style="list-style-type: none"> Check that the correct wash buffer is being used.
	Improper reagent preparation	<ul style="list-style-type: none"> Consult reagent preparation section for the correct dilutions of all reagents.

	Insufficient or prolonged incubation periods	<ul style="list-style-type: none"> Consult the provided procedure for correct incubation time.
Deficient Standard Curve Fit	Non-optimal sample dilution	<ul style="list-style-type: none"> Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay. Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay. User should determine the optimal dilution factor for samples.
	Contamination of reagents	<ul style="list-style-type: none"> A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporate	<ul style="list-style-type: none"> Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature.
	Improper pipetting	<ul style="list-style-type: none"> Pipette properly in a controlled and careful manner. Check pipette calibration. Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	<ul style="list-style-type: none"> Thoroughly agitate the lyophilized components after reconstitution. Thoroughly mix dilutions.

References

- (1) Lundwall A *et al.* (1986) *Proc Natl Acad Sci USA*. 83:6716-6720.
- (2) Hoskins J *et al.* (1986) *Proc Natl Acad Sci USA*. 84:349-353.
- (3) Suzuki K *et al.* (1983) *J Biochem*. 94:699-705.
- (4) Dahlback B, Stenflo J. (1981) *Proc Natl Acad Sci USA*. 78:2512-2516.
- (5) Webb JH *et al.* (2003) *Blood Coagul Fibrinolysis*. 14(4):355-359.
- (6) Kask L *et al.* (2004) *J Biol Chem*. 279(23):23869-23873.

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