AssayMax™

Human Complement C4 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 µl of Standard or Sample per well.
Incubate 2 hours.

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

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

Step 4. Wash, then add 50 µl of Chromogen Substrate per well.
Incubate 10 minutes.

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

Symbol Key

Consult instructions for use.

Assay Template

	A	B	C	D	E	F	G	H
1								
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Human Complement C4 ELISA Kit

Catalog No. EC3202-1

Sample insert for reference use only

Introduction

Complement component 4 (C4) plays a key role in the activation of the classical complement pathway. C4 is synthesized as a single-chain precursor molecule (200 kDa) but processed to the three-chain disulphide-linked structure with alpha (93 kDa), beta (78 kDa), and gamma (33 kDa) chains prior to secretion (1-3). After activation by C1s, C4 is processed to C4a and C4b. C4a anaphylatoxin is a mediator of local inflammation and induces smooth muscle contraction (4). C4b, the major activation product, is an essential subunit of the C3 and C5 convertases of the classical complement pathway.

Principle of the Assay

The AssayMax Human Complement C4 ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for detection of human complement C4 in **urine, milk, saliva, CSF, and cell culture samples**. This assay employs a quantitative **sandwich enzyme immunoassay** technique that measures human complement C4 in less than 4 hours. A polyclonal antibody specific for human complement C4 has been pre-coated onto a 96-well microplate with removable strips. Complement C4 in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for complement C4, which is recognized by a streptavidin-peroxidase 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 For Use In Diagnostic Procedures.
- Prepare all reagents (working 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 Complement C4 Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human complement C4.
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human Complement C4 Standard:** Human complement C4 in a buffered protein base (90 ng, lyophilized).
- **Biotinylated Human Complement C4 Antibody (50x):** A 50-fold concentrated biotinylated polyclonal antibody against complement C4 (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).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 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.
- Diluent (1x) may be stored for up to 30 days at 2-8°C.
- 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 µl, 20-200 µl, 200-1000 µl, and multiple channel).

- Deionized or distilled reagent grade water.

Sample Collection, Preparation, and Storage

- **Urine:** Collect urine using sample pot. Centrifuge samples at 800 x *g* for 10 minutes. Dilute samples 1:2 into MIX Diluent and assay. 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 pot. Centrifuge samples at 800 x *g* for 10 minutes. Dilute samples 1:200 into MIX Diluent and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 3000 x *g* for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.
- **Milk:** Collect milk using sample tube. Centrifuge samples at 800 x *g* for 10 minutes. Milk dilution is suggested at 1:8000 into MIX Diluent; however, the user should determine the optimal dilution factor. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **CSF:** Collect cerebrospinal fluid (CSF) using sample pot. Centrifuge samples at 3000 x *g* for 10 minutes. Dilute samples 1:500 into MIX Diluent and assay. If necessary, further dilute samples. The undiluted samples can be stored at -80°C for up to 3 months. Avoid repeated freeze-thaw cycles.

Refer to Sample Dilution Guidelines below for further instruction.

Guidelines for Dilutions of 1:100 or Greater
(*for reference only; please follow the insert for specific dilution suggested)*

1:100	**1:10000**
A) 4 ul 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.*
1:1000	**1:100000**
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.
- **MIX Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent Concentrate 1:10 with reagent grade water. Store for up to 30 days at 2-8°C.
- **Standard Curve:** Reconstitute the 90 ng of Human Complement C4 Standard with 4.5 ml of MIX Diluent to generate a 20 ng/ml standard stock solution. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the standard stock solution (20 ng/ml) 1:4 with MIX Diluent to produce 5, 1.25, 0.3125, and 0.0781 ng/ml solutions. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within 30 days.

Standard Point	Dilution	[Complement C4] (ng/ml)
P1	1 part Standard (20 ng/ml)	20.000
P2	1 part P1 + 3 parts MIX Diluent	5.0000
P3	1 part P2 + 3 parts MIX Diluent	1.2500
P4	1 part P3 + 3 parts MIX Diluent	0.3125
P5	1 part P4 + 3 parts MIX Diluent	0.0781
P6	MIX Diluent	0.0000

- **Biotinylated Human Complement C4 Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:50 with MIX Diluent. Any remaining solution should be frozen at -20°C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen 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.

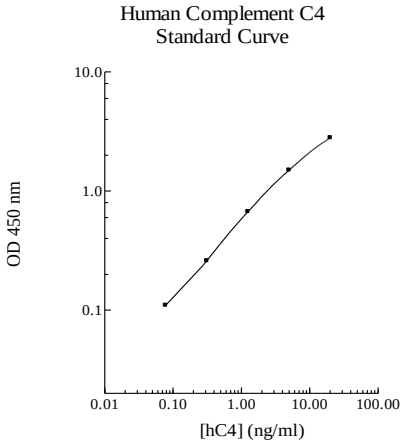
- Add 50 µl of Human Complement C4 Standard or sample per well. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material 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.
- Add 50 µl of Biotinylated Human Complement C4 Antibody to each well and incubate for 1 hour.
- Wash the microplate as described above.
- Add 50 µl of Streptavidin-Peroxidase Conjugate to each well 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 µl of Chromogen Substrate per well and incubate for 10 minutes or till the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
- 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 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.



Performance Characteristics

- The minimum detectable dose of C4 is typically ~ 0.07 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.8% and 7.1% respectively.

Recovery

Standard Added Value	0.3 – 3.0 ng/ml
Recovery %	88 – 109%
Average Recovery %	98%

Linearity

Average Percentage of Expected Value (%)	
Sample Dilution	Milk
1:4000	98%
1:8000	99%
1:16000	104%

Cross-Reactivity

Species	Cross Reactivity (%)
Canine	<5%
Bovine	None
Monkey	<10%
Mouse	None
Rat	None
Swine	None
Rabbit	None

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 dry 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 lyophilized components 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.

Deficient Standard Curve Fit	Non-optimal sample dilution	• 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	• A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporate	• Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature.
	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 lyophilized components after reconstitution. • Thoroughly mix dilutions.

References

(1) Roos MH *et al.* (1982) *Nature* 298(5877):854-856
(2) Miura N *et al.* (1987) *J Biol. Chem.* 262(15):7298-7305
(3) Belt KT *et al.* (1984) *Cell* 36(4):907-914
(4) Moon KE *et al.* (1981) *J Biol Chem.* 256(16):8685-8692

Version 2.4R

Related Products

- EC1101-1 AssayMax Human Complement C1q ELISA Kit (Plasma, Serum, Urine, Milk, Saliva, and Cell Culture samples)
- EC1102-1 AssayMax Human Complement C1r ELISA Kit (Plasma, Serum, Urine, Milk, Saliva, and Cell Culture samples)
- EC1111-1 AssayMax Human Complement C1 ELISA Kit (Plasma, Serum, Urine, Milk, Saliva, and Cell Culture samples)
- EC2001-1 AssayMax Human Complement C2 ELISA Kit (Plasma, Serum, Saliva, and Cell Culture samples)
- EC2101-1 AssayMax Human Complement C3 ELISA Kit (Plasma and Serum samples)
- EC3201-1 AssayMax Human Complement C3 ELISA Kit (Urine, Saliva, Milk, and Cell Culture samples)
- EC3301-1 AssayMax Human Complement C3b ELISA Kit (Plasma, Serum, Urine, Milk, Saliva, CSF, and Cell Culture samples)
- EC2102-1 AssayMax Human Complement C4 ELISA Kit (Plasma and Serum samples)
- EC2202-1 AssayMax Human Complement C4BP ELISA Kit (Plasma, Serum, Urine, Saliva, Milk, CSF, and Cell Culture samples)
- EC5101-1 AssayMax Human Complement C5 ELISA Kit (Plasma, Serum, Milk, Saliva, and Cell Culture samples)
- EC6101-1 AssayMax Human Complement C6 ELISA Kit (Plasma, Serum, Milk, Saliva, Urine, and Cell Culture samples)
- EC7101-1 AssayMax Human Complement C7 ELISA Kit (Plasma, Serum, Milk, Saliva, Urine, and Cell Culture samples)
- EC8101-1 AssayMax Human Complement C8 ELISA Kit (Plasma, Serum, Urine, Milk, Saliva, and Cell Culture samples)
- EC9101-1 AssayMax Human Complement C9 ELISA Kit (Plasma, Serum, Urine, Milk, Saliva, and Cell Culture samples)