AssayLite™

Human Complement C4 Fluorescent Immunoassay Kit

(Red Fluorescent Probe)

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This product is manufactured under patented technology by Assaypro LLC

US Patent No. 9,945,847

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

Thank you for choosing Assaypro.

Table of Contents

Assay Summary

Step 1. Add 50 µl of Standard/Sample and 50 µl of Fluorescent Probe per well.
Incubate 45 minutes at 37°C.

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

Step 3. Read at EX 485/20 nm, EM 575/15 nm

Symbol Key

Consult instructions for use.

Assay Mechanism

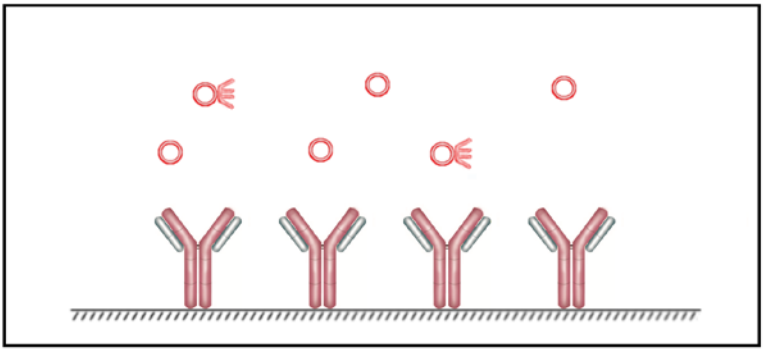

Figure 1. Standard, samples, and the fluorescent probe are added to wells and incubated. The fluorescent probe competes for binding sites with the standard and samples.

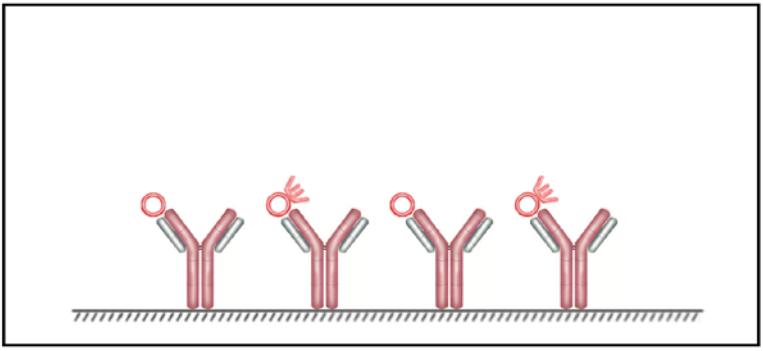

Figure 2. The microplate is washed and the endpoint fluorescence is measured. The fluorescence intensity is inversely proportional to the concentration of the Antigen A in the standard or samples.

Assay Template

	A	B	C	D	E	F	G	H
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AssayLite™ Human Complement C4 Fluorescent Immunoassay Kit

Catalog No. FEC2102

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. C4 deficiency is associated with systemic lupus erythematosus (5). The C4b degradation product, C4d, is a marker for humoral rejection in allografts (6).

Principle of the Assay

The AssayLite™ Human Complement C4 Fluorescent Immunoassay Kit employs a **quantitative competitive fluorescent probe technique** that measures complement C4 in human **plasma, serum, saliva, milk, and cell culture samples** in approximately 45 minutes. A polyclonal antibody specific for human complement C4 has been pre-coated onto a 96-well opaque microplate with removable strips. Human complement C4 in standards and samples is competed with a human complement C4 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 probe) 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 probe in a dark place. Do not freeze.

Reagents

- **Human Complement C4 Microplate:** A 96-well opaque 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 aluminum 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 (86.4 µg, lyophilized).
- **Red Fluorescent Human Complement C4 Probe (5x):** Lyophilized.
- **EIA Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml).
- **Stabilizing Solution (1x)**: A solution to stabilize the fluorescent component (8 ml).

Storage Condition

- 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 desiccants 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 Probe 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

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 320-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 (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 320-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. The sample is suggested for use at 1x; 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.
- **Milk:** Collect milk 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.

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 Complement C4 Standard:** Reconstitute the Human Complement C4 Standard (86.4 μg) with 1.8 ml of EIA Diluent to generate a 48 μg/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 (48 μg/ml) 4-fold with EIA Diluent to produce 12, 3, 0.75, 0.188, and 0.047 μg/ml solutions. 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	[Complement C4] (μg/ml)
P1	1 part Standard (48 μg/ml)	48
P2	1 part P1 + 3 parts EIA Diluent	12
P3	1 part P2 + 3 parts EIA Diluent	3
P4	1 part P3 + 3 parts EIA Diluent	0.75
P5	1 part P4 + 3 parts EIA Diluent	0.188
P6	1 part P5 + 3 parts EIA Diluent	0.047
P7	EIA Diluent	0

- **Red Fluorescent Human Complement C4 Probe (5x):** Reconstitute the fluorescent probe with 1 ml of EIA Diluent to generate a stock solution. Allow the vial to sit for 10 minutes with gentle agitation prior to dilution. From the stock solution, dilute 5-fold with EIA Diluent to produce a 1x working solution. Any remaining stock solution should be stored at 2-8°C and used within 7 days. Do not freeze.
- **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

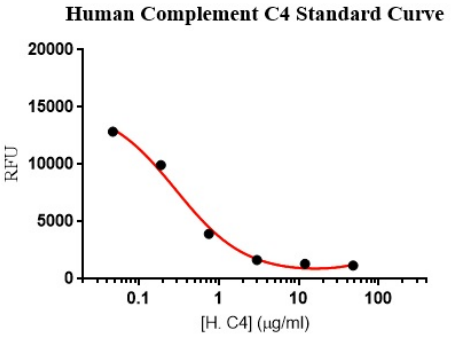
- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is incubated at 37°C.
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Human Complement C4 Standard or sample to each well, and immediately add 50 µl of Red Fluorescent Human Complement C4 Probe 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 tape and incubate for 45 minutes. 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 µl of Wash Buffer per well; invert the plate and 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.
- Read the endpoint fluorescence on a microplate reader at an excitation wavelength of 485/20 nm and emission wavelength of 575/15 nm.
- For the Synergy H1F, a gain of 75 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 Complement C4 Average Red RFU
BioTek – Synergy H1F	P1	48	1168
	P2	12	1318
	P3	3	1654
	P4	0.75	3944
	P5	0.188	9944
	P6	0.047	12860
	P7	0	13443

- The minimum detectable dose of human complement C4 as calculated by 2SD from the mean of a zero standard was established to be 0.045 µg/ml.
- Intra-assay precision was determined by testing three plasma samples twenty times in one assay.
- Intra-assay and inter-assay coefficients of variation were 4.0% and 10.1% respectively.

Reference Value

- Normal human complement C4 plasma levels range from 160 – 480 µg/ml.
- Plasma and serum samples from healthy adults were tested (n=40). On average, human complement C4 level was 302 µg/ml.

Sample	n	Average Value (µg/ml)
Pooled Normal Plasma	10	296
Normal Plasma	20	284
Pooled Normal Serum	10	327

Recovery

Standard Added Value	0.5 – 5 µg/ml
Recovery %	85 – 110%
Average Recovery %	97.5%

Linearity

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

Average Percentage of Expected Value (%)		
Sample Dilution	Plasma	Serum
160x	98%	100%
320x	95%	97%
640x	107%	103%

Cross-Reactivity

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

Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of improper 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

(1) Roos MH *et al.* (1982) *Nature.* 298(5877):854-856.
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(3) Belt KT *et al.* (1984) *Cell.* 36(4):907-914.
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(6) Girnita AL *et al.* (2008) *Transplantation.* 86(2):342-347.

Version 1.0