



BD OptEIA™

Human C5a ELISA Kit II

Instruction Manual

Catalog No. 557965



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Introduction

Activation of the classical, alternate, or lectin complement pathways can result in the production of the C5a anaphylatoxin.^{1,2} C5a is a multifunctional proinflammatory mediator. Thus, C5a has been observed to increase vascular permeability, to be spasmogenic and chemotactic, and to induce the release of pharmacologically active mediators from a number of cell types.³ C5a production *in vivo* may also initiate, contribute to, or exacerbate the inflammatory reactions seen in gram-negative bacterial sepsis, trauma, ARDS, ischemic heart disease, post-dialysis syndrome, and several autoimmune diseases including rheumatoid arthritis, lupus erythematosus, and acute glomerulonephritis.

In blood plasma or serum, once formed, the nascent C5a anaphylatoxin is rapidly cleaved to the C5a-desArg form by the endogenous serum carboxypeptidase N enzyme.⁴ Thus, the quantitation of C5a-desArg in plasma or experimental samples should yield a reliable measurement of the level of complement activation that has occurred in the test samples under investigation.

The BD OptEIA™ Human C5a ELISA Kit II is for the *in vitro* quantitative determination of human C5a-desArg in human EDTA plasma, serum and other biological samples.

Principle of the Test

The BD OptEIA™ ELISA test is a solid phase sandwich ELISA (Enzyme-Linked Immunosorbent Assay). It utilizes monoclonal antibody specific for human C5a-desArg coated on a 96-well plate. Standards and samples are added to the wells, and any C5a-desArg present binds to the immobilized antibody. The wells are washed and a mixture of biotinylated anti-human C5a antibody and streptavidin-horseradish peroxidase is added, producing an antibody-antigen-antibody “sandwich”. The wells are again washed and a substrate solution is added, which produces a blue color in direct proportion to the amount of C5a-desArg present in the initial sample. The Stop Solution changes the color from blue to yellow, and the wells are read at 450 nm.

Reagents Provided

Antibody Coated Wells:	1 plate of 96 breakable wells (12 strips × 8 wells) coated with anti-human C5a-desArg monoclonal antibody
Detection Antibody:	15 mL of biotinylated anti-human C5a monoclonal antibody containing fetal bovine serum* and ProClin™-150 as preservative
Standards:	3 vials lyophilized human serum containing a defined amount of C5a-desArg (quantity as noted on vial label)
Enzyme Concentrate (250×):	150 µL of concentrated Streptavidin-horseradish peroxidase conjugate with BSA* and ProClin™-150 as preservative
Standard/Sample Diluent:	30 mL of animal serum* with 0.09% sodium azide as preservative
ELISA Diluent:	12 mL of a buffered protein base with 0.09% sodium azide as preservative
Wash Concentrate (20×):	100 mL of 20× concentrated detergent solution with ProClin™-150
TMB One-Step Substrate Reagent:	30 mL of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution
Stop Solution:	13 mL of 1 M phosphoric acid
Plate Sealers:	2 sheets with adhesive backing

**Source of all serum proteins is from USDA inspected abattoirs located in the United States.*

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Materials Required but not Provided

- Microplate reader capable of measuring absorbance at 450 nm
- Precision pipettes to deliver 50 μ L and 100 μ L volumes
- Adjustable 1 mL, 5 mL, 10 mL, 25 mL pipettes for reagent preparation
- Deionized or distilled water
- Wash bottle or automated microplate washer
- Log-log graph paper or automated data reduction
- Tubes to prepare standard dilutions
- Laboratory timer
- Absorbent paper
- FUT-175 (Futhan) protease inhibitor and plasma stabilizer for complement measurements (Cat. No. 552035) recommended

Storage Information

1. Store unopened kit at 2 - 8°C. Do not use kit after expiration date.
2. Before use, bring all reagents to room temperature (18 - 25°C). Immediately after use, return to proper storage conditions.
3. Lyophilized standards are stable until kit expiration date. After reconstitution, use freshly reconstituted standard within 12 hours (stored at 2 - 8°C).

Warnings and Precautions

1. Reagents which contain preservatives may be toxic if ingested, inhaled, or come in contact with skin.
2. Avoid contact of skin, eyes, or clothing with Stop Solution or Substrate Reagents.
3. Handle all serum and plasma specimens in accordance with NCCLS guidelines for preventing transmission of blood-borne infections.
4. Standard/Sample Diluent and ELISA Diluent contain less than 0.1% sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

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5. **Warning**

Wash Concentrate (20X) (component 51-9003738) contains 0.002% (w/w), Human C5a Lyophilized Standard (component 51-9004473) contains 0.02% (w/w), and Detection Antibody Biotin Anti-Human C5a (component 51-27122E) contains 0.003% (w/w) of a CMIT/MIT mixture (3:1), which is a mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC No 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC No 220-239-6] (3:1).

Hazard statements

May cause an allergic skin reaction.

Precautionary statements

Wear protective gloves /eye protection.

Wear protective clothing.

Avoid breathing mist/vapours/spray.

If skin irritation or rash occurs: Get medical advice/attention.

IF ON SKIN: Wash with plenty of water.

Dispose of contents/container in accordance with local/regional/national/international regulations.

6. **Danger**

Stop Solution (component 51-2608KC) contains 15.23% phosphoric acid (w/w).

Hazard statements

Causes severe skin burns and eye damage.

Precautionary statements

Wear protective gloves / eye protection.

Wear protective clothing.

IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.

IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do.

Continue rinsing.

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IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing.

Dispose of contents/container in accordance with local/regional/national/international regulations.

7. This product contains human blood, serum, cells, or materials derived from them, which are potentially hazardous materials. Use universal precautions when handling. Handle as if product were capable of transmitting disease. Material used in this product has been tested using FDA approved methods and found negative for Human Immunodeficiency Virus (HIV-1/HIV-2). Hepatitis B Surface Antigen (HBsAg) and antibody to Hepatitis C Virus (HCV). However, no known test method can offer complete assurance that specimens of human origin will not transmit infectious disease. When handling or disposing, follow precautions described in CDC and FDA recommendations and OSHA Bloodborne Pathogen recommendations.

Specimen Collection and Handling

Specimens should be clear, non-hemolyzed and non-lipemic. Samples with expected values higher than the top standard (5 ng/mL) should be diluted (ie, 1:4, 1:10) with Standard/Sample Diluent prior to running the assay.

Note: Handle and dispose of all specimens as if they are capable of transmitting infectious agents. It is critical that sample collection is performed correctly. Care must be taken to avoid C5a generation in the samples. All specimen handling operations should be carried out at 4°C for plasma and for serum (immediately after clotting).

Plasma: Collect plasma using disodium EDTA as the anticoagulant. If possible, collect the plasma into a mixture of disodium EDTA and FUT-175 (Futhan, BD Cat. No. 552035) to stabilize the sample against spontaneous *in vitro* complement activation. Immediately centrifuge samples at 4° C for 15 minutes at 1000 x g. Assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of plasma may also be stored at -70° C for extended periods of time. Avoid repeated freeze-thaw cycles.

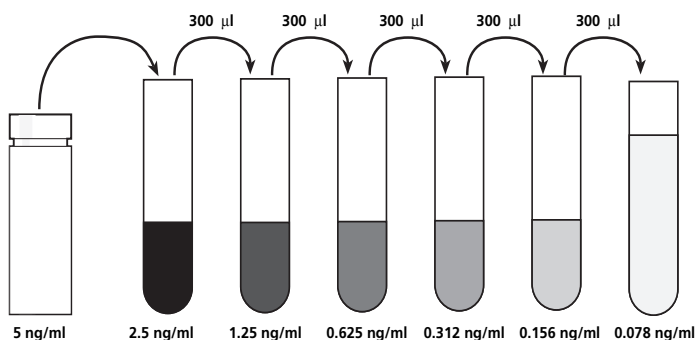
Serum: Use a serum tube (eg, BD Vacutainer® Cat. No. 366430) and allow samples to clot for 60 ± 30 minutes. Centrifuge the samples at 4°C for 10 minutes at 1000 x g. Remove serum and assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of serum may also be stored at -70° C for extended periods of time. Avoid repeated freeze-thaw cycles.

Other biological samples: Remove any particulate matter by centrifugation and assay immediately or store samples at -70° C. Avoid repeated freeze/thaw cycles.

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Reagent Preparation

1. Bring all reagents to room temperature (18 - 25°C) before use.
2. Standards
 - a. Reconstitute 1 vial lyophilized Standard with required volume (noted on vial label) of Standard Diluent to prepare a 5 ng/mL stock standard. Allow the standard to equilibrate for at least 15 minutes before making dilutions. Vortex to mix.
 - b. Add 300 µL Standard Diluent to 6 tubes. Label as 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0.3125 ng/mL, 0.156 ng/mL, and 0.078 ng/mL.
 - c. Perform serial dilutions by adding 300 µL of each standard to the next tube and vortexing between each transfer. The undiluted standard serves as the high standard (5 ng/mL). The Standard Diluent serves as the zero standard (0 ng/mL).



3. Working Detector
See *Assay Procedure*, step 5.

4. Wash Buffer

Note: If the Wash Concentrate contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute required quantity of 20× Wash Concentrate with deionized or distilled water, mix. (To prepare 2,000 mL, add 100 mL Wash Concentrate to 1,900 mL water. At least 500 mL solution should be prepared for a full 96-well plate).

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5. TMB One-Step Substrate Reagent

No more than 15 minutes prior to use, add required volume of TMB One-Step Substrate Reagent to a clean tube or reservoir. To prevent contamination, pipette out from the tube/reservoir instead of directly from bottle. Avoid prolonged exposure to light or contact with metal, air, or extreme temperature as color may develop.

Assay Procedure

1. Bring all reagents and samples to room temperature (18 - 25°C) prior to use. It is recommended that all standards and samples be run in duplicate. A standard curve is required in each assay run.
2. Place required quantity of test strips/wells in well holder.

Note: Wells are provided in breakable 8-well strips. Strips may be “broken” into individual wells, replaced in well holder, and assayed. Return any unused wells to sealed pouch for 2 - 8°C storage.
3. Pipette 50 µL of ELISA Diluent into each well.
4. Pipette 100 µL of each standard (see *Reagent Preparation*, step 2) and sample into appropriate wells. Gently shake/tap the plate for 5 seconds to mix. Cover wells with Plate Sealer and incubate for 2 hours at room temperature
5. Prepare Working Detector. Within 15 minutes prior to use, pipette required volume of Detection Antibody into a clean tube or flask. Add in required quantity of Enzyme Concentrate (250×), vortex or mix well. For a full 96-well plate, add 48 µL of Enzyme Concentrate into 12 mL of Detection Antibody.
6. Decant or aspirate contents of wells. Wash wells by filling with at least 300 µL/well prepared Wash Buffer (see *Reagent Preparation*, step 4) and then decanting/aspirating. Repeat wash 4 times for a total of 5 washes. After the last wash, blot plate on absorbent paper to remove any residual buffer. Complete removal of liquid is required for proper performance.
7. Add 100 µL of prepared Working Detector (see *step 5* above) to each well. Gently shake/tap the plate for 5 seconds to mix. Cover wells with Plate Sealer and incubate for 1 hour at room temperature.

8. Wash wells as in Step 6, but a total of 7 times.

Note: In this final wash step, soak wells in wash buffer for 30 seconds to 1 minute for each wash. Thorough washing at this step is very important.

9. Add 100 μL of TMB One-Step Substrate Reagent to each well. Gently shake/tap the plate for 5 seconds to mix. Incubate plate (without Plate Sealer) for 30 minutes at room temperature in the dark.
10. Add 50 μL of Stop Solution to each well. Gently shake/tap the plate for 5 seconds to mix.
11. Read absorbance at 450 nm within 30 minutes of stopping reaction. If wavelength correction is available, subtract A (570 nm) from A (450 nm).

Assay Procedure Summary

1. Add 50 μL ELISA Diluent to each well.
2. Add 100 μL standard or sample to each well.
Incubate 2 hours at room temperature.
3. Aspirate and wash 5 times.
4. Add 100 μL prepared Working Detector to each well.
Incubate 1 hour at room temperature.
5. Aspirate and wash/soak 7 times.
6. Add 100 μL TMB Substrate Reagent to each well.
Incubate 30 minutes at room temperature.
7. Add 50 μL Stop Solution to each well.
Read at 450 nm within 30 minutes.
 λ correction 570 nm.

Calculation of Results

Calculate the mean absorbance for each set of duplicate standards, controls and samples. Subtract the mean zero standard absorbance from each.

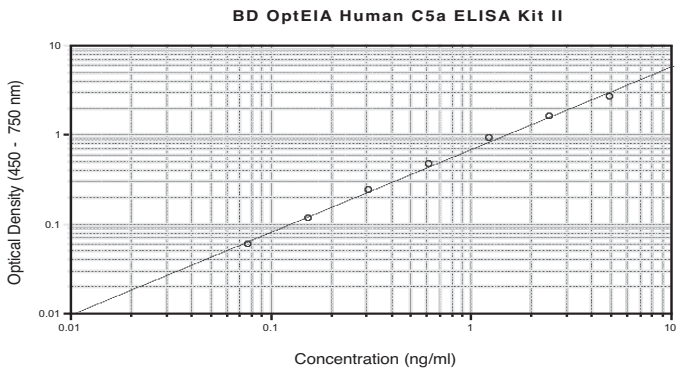
Plot the standard curve on log-log graph paper, with C5a-desArg concentration on the x-axis and absorbance on the y-axis. Draw the best fit straight line through the standard points.

To determine the C5a-desArg concentration of the unknowns, find the unknowns' mean absorbance value on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the C5a-desArg concentration. If samples were diluted, multiply the C5a-desArg concentration by the dilution factor.

Computer curve-fitting statistical software may also be employed.

Typical Data

This standard curve is for demonstration only. A standard curve must be run with each assay.



Concentration (ng/mL)	OD1	OD2	Mean	Zero Standard Subtracted
0	0.032	0.031	0.032	0.000
0.078	0.091	0.078	0.085	0.053
0.156	0.149	0.136	0.143	0.111
0.312	0.264	0.263	0.264	0.232
0.625	0.498	0.483	0.491	0.459
1.25	0.949	0.928	0.939	0.907
2.5	1.582	1.605	1.594	1.562
5.0	2.611	2.610	2.611	2.579

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Limitations of the Procedure

- 1. This kit is intended for use as an integral unit. Do not mix reagents from different kit lots. Reagents from other manufacturers or other antibodies should not be used in this kit.
- 2. Interference by drug metabolites, soluble receptors, or other binding proteins in specimens has not been thoroughly investigated. The possibility of interference cannot be excluded

Performance

Limit of Detection

The minimum detectable dose of C5a was determined to be 0.047 ng/mL. This is defined as two standard deviations above the mean optical density of 20 replicates of the zero standard.

Recovery

Three different levels of Human C5a were spiked into 6 samples each of various matrices. Results are compared with same amounts of human C5a spiked into Standard Diluent, as follows:.

Samples	No. of Samples (n)	Quantity Spiked (ng/mL)	Average % Recovery	Range
Serum	7	20	101.9	95.7 - 106.5
	7	10	96.0	85.0 - 99.4
	7	5	97.7	93.7 - 101.1
Plasma	8	20	95.6	88.4 - 107.9
	8	10	101.3	96.8 - 103.1
	8	5	105.8	93.0 - 115.2
Cell culture media	3	20	111.5	104.8 - 118.6
	3	10	108.7	99.5 - 115.7
	3	5	111.7	103.5 - 117.3

Linearity

Samples spiked with high concentrations of human C5a were serially diluted with Standard Diluent and run in the BD OptEIA human C5a Kit II. Results are as follows:

Samples	No. of Samples (n)	Quantity Spiked	Average % Recovery	Range
Serum	3	1:2	108.3	101.4 - 112.1
	3	1:4	97.2	85.8 - 104.2
	3	1:8	92.7	82.9 - 100.9
	3	1:16	77.9	71.3 - 81.9
Plasma	6	1:2	100.3	93.3 - 112.4
	6	1:4	100.1	87.5 - 114.3
	6	1:8	98.4	85.4 - 120.3
	6	1:16	92.9	82.2 - 13.2
Cell culture media	6	1:2	99.9	86.6 - 111.0
	6	1:4	98.2	87.8 - 111.2
	6	1:8	98.4	81.4 - 114.3
	6	1:16	89.0	77.6 - 111.8

Specificity

Cross-Reactivity

The following factors were tested in the BD OptEIA human C5a ELISA Kit II. Cross reactivity is reported as% by weight.

Human C3a	Not Detectable (<0.00078)
Human C4a	0.015
Human C3	Not Detectable (<0.00078)
Human C4	Not Detectable (<0.00078)
Human C5	0.048

Precision

Intra-assay

Twenty-four replicates each of three different levels of human C5a were tested in one plate. The following results were observed:

Number of replicates	24	24	24
Mean Concentration	2.37 ng/mL	1.27 ng/mL	0.067 ng/mL
SD	0.060	0.052	0.031
%CV	2.5	4.1	4.6

Inter-assay

Three different levels of C5a-desArg were tested in four different assays. The following results were observed:

Number of replicates	32	32	32
Mean Concentration	2.38 ng/mL	1.26 ng/mL	0.68 ng/mL
SD	0.122	0.068	0.054
%CV	5.1	5.4	7.9

Standardization

This immunoassay is calibrated against purified Human C5a-desArg.

Experimental Results

Serum/ Plasma

Fifteen serum samples were tested in this assay. The mean C5a-desArg value was 39.65 ng/mL, with a range from 11.48 to 77.78 ng/mL.

Nineteen plasma (EDTA) samples were tested in this assay. The mean C5a-desArg value was 15.94 ng/mL, with a range from 5.01 to 30.46 ng/mL.

Cobra Venom Factor-Activated Human Serum

A pooled CVF-activated human serum sample was tested in this kit and demonstrated a C5a-desArg value of 3,422.59 ng/mL.

Aggregated IgG Activated Serum

A pooled aggregated IgG-activated serum sample was tested in this kit and demonstrated a C5a-desArg value of 1,617.08 ng/mL.

Troubleshooting

Problem	Possible Source	Corrective Action
Poor Precision	<ul style="list-style-type: none"> • Inadequate washing / aspiration of wells • Inadequate mixing of reagents • Imprecise / inaccurate pipetting • Imprecise sealing of plate 	<ul style="list-style-type: none"> • Check function of washing system • Ensure adequate mixing • Check / calibrate pipettes • Ensure complete sealing of plate
Poor Standard Curve	<ul style="list-style-type: none"> • Improper standard handling / dilution • Incomplete washing / aspiration of wells • Imprecise / inaccurate pipetting 	<ul style="list-style-type: none"> • Ensure correct preparation of standards • Check function of washing system • Check / calibrate pipettes
Low Signal	<ul style="list-style-type: none"> • Inadequate reagent volumes added to wells • Incorrect incubation times / temperature • Overly high wash / aspiration pressure from automated plate-washer. 	<ul style="list-style-type: none"> • Check / calibrate pipettes • Ensure sufficient incubation times / reagents warmed to room temperature • Utilize manual washing

References

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Plate Templates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
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	1	2	3	4	5	6	7	8	9	10	11	12
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