Human C5a ELISA Kit

Enzyme-linked Immunosorbent Assay for quantitative detection of human C5a

Catalog Numbers BMS2088 or BMS2088TEN

Pub. No. MAN0016533 **Rev.** A.0 (30)

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Human C5a ELISA Kit is an enzyme-linked immunosorbent assay for the quantitative detection of human C5a.

Summary

C5a is a protein fragment released from cleavage complement component C5 by protease C5-convertase into C5a and C5b fragments. C5b is important in late events of complement cascade, whereas C5a acts as highly inflammatory peptide.

The origin of C5 is in the hepatocyte but its synthesis can also be found in macrophages that may cause local increase of C5a. C5a has chemotactic and anaphylatoxic properties, it is essential in the innate immunity but it is also linked with the adaptive immunity. The increase production of C5a is connected with a number of inflammatory diseases.

C5a interact with receptor protein C5a1, C5aR or CD88 on the surface of target cells such as macrophages, neutrophils and endothelial cells.

For literature update refer to our website.

Principles of the test

An anti-human C5a coating antibody is adsorbed onto microwells.

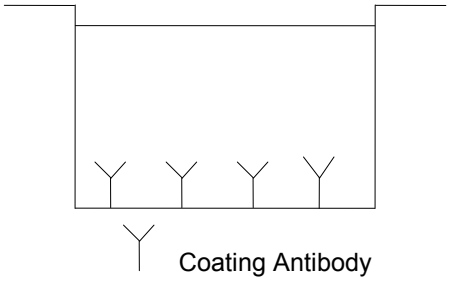

Fig. 1 Coated microwell

Human C5a present in the sample or standard binds to antibodies adsorbed to the microwells.

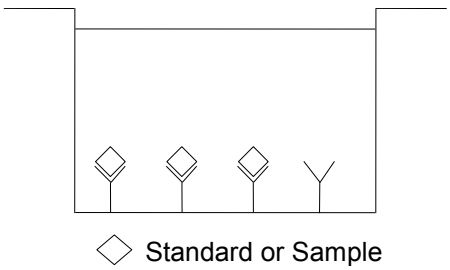

Fig. 2 First incubation

Following incubation unbound biological components are removed during a wash step and a biotin-conjugated anti-human C5a antibody is added and binds to human C5a captured by the first antibody.

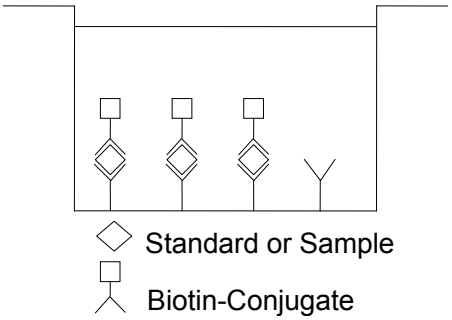

Fig. 3 Second incubation

Following incubation unbound biotin-conjugated anti-human C5a antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-human C5a antibody.

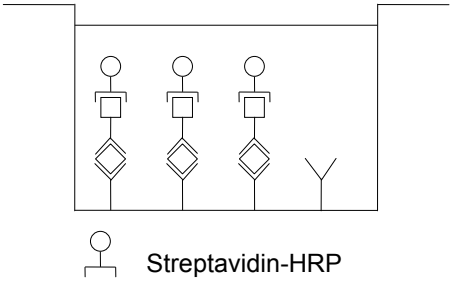

Fig. 4 Third incubation

Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

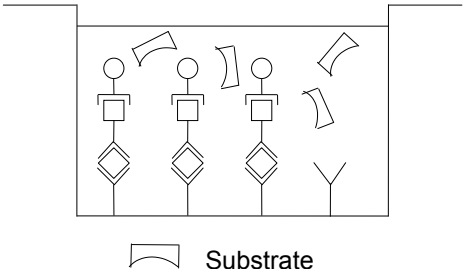

Fig. 5 Fourth incubation

A colored product is formed in proportion to the amount of human C5a present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven human C5a standard dilutions and human C5a sample concentration determined.

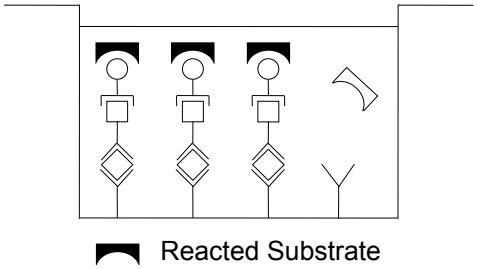

Fig. 6 Stop reaction

Reagents provided

Reagents for human C5a ELISA BMS2088 (96 tests)

1 aluminum pouch with a Microwell Plate (12 strips with 8 wells each) coated with monoclonal antibody to human C5a

1 vial (120 µL) Biotin-Conjugate anti-human C5a monoclonal antibody

1 vial (150 µL) Streptavidin-HRP

2 vials human C5a Standard lyophilized, 5 ng/mL upon reconstitution

Note: In some, very rare cases, an insoluble precipitate of stabilizing protein has been seen in the vial. This precipitate does not interfere in any way with the performance of the test and can thus be ignored.

1 vial (50 mL) Sample Diluent

1 vial (5 mL) Calibrator Diluent

1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (15 mL) Stop Solution (1M Phosphoric acid)

6 Adhesive Films

Reagents for human C5a ELISA BMS2088TEN (10x96 tests)

10 aluminum pouches with a Microwell Plate (12 strips with 8 wells each) coated with monoclonal antibody to human C5a

10 vials (120 µL) Biotin-Conjugate anti-human C5a monoclonal antibody

10 vials (150 µL) Streptavidin-HRP

10 vials human C5a Standard lyophilized, 5 ng/mL upon reconstitution

Note: In some, very rare cases, an insoluble precipitate of stabilizing protein has been seen in the vial. This precipitate does not interfere in any way with the performance of the test and can thus be ignored.

6 vials (50 mL) Sample Diluent

5 vials (5 mL) Calibrator Diluent

3 vials (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

10 bottles (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

10 vials (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (100 mL) Stop Solution (1M Phosphoric acid)

30 Adhesive Films

Storage instructions – ELISA kit

Store kit reagents between 2°C and 8°C. Immediately after use remaining reagents should be returned to cold storage (2°C to 8°C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

Sample collection and storage instruction

Cell culture supernatant, serum, and plasma (EDTA, citrate, heparin) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic samples.

Samples should be aliquoted and must be stored frozen at –20°C to avoid loss of bioactive human C5a. If samples are to be run within 24 hours, they may be stored at 2–8°C (for sample stability refer to “Specificity“ on page 6).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Materials required but not provided

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microplate shaker
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

Precautions for use

- All reagents should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipet by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or samples.
- Rubber or disposable latex gloves should be worn while handling kit reagents or samples.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- To avoid microbial contamination or cross-contamination of reagents or samples that may invalidate the test, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.

- Decontaminate and dispose samples and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

Preparation of reagents

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

Wash buffer (1x)

1. Pour entire contents (50 mL) of the Wash Buffer Concentrate (20x) into a clean 1000 mL graduated cylinder. Bring to final volume of 1000 mL with glass-distilled or deionized water. Mix gently to avoid foaming.
2. Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.
3. Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

Assay buffer (1x)

1. Pour the entire contents (5 mL) of the Assay Buffer Concentrate (20x) into a clean 100 mL graduated cylinder. Bring to final volume of 100 mL with distilled water. Mix gently to avoid foaming.
2. Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.
3. Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

Biotin-Conjugate

Note: The Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (mL)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

Streptavidin-HRP

Note: The Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:200 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

Human C5a standard

1. Reconstitute human C5a standard by addition of Calibrator Diluent (for subsequent measurement of serum or plasma samples) or Sample Diluent (for subsequent measurement of cell culture supernatant samples).
2. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 5 ng/mL).
3. Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.
4. The standard has to be used immediately after reconstitution and cannot be stored.

External standard dilution

1. Label 7 tubes, one for each standard point: S2, S3, S4, S5, S6, S7.

   The reconstituted human C5a standard serves as S1.
2. Prepare 1:2 serial dilutions for the standard curve as follows: Pipette 150 µL of Calibrator Diluent (for subsequent measurement of serum or plasma samples) or Sample Diluent (for subsequent measurement of cell culture supernatant samples) into each tube.
3. Pipette 150 µL of reconstituted standard (concentration = 5 ng/mL) into the tube, labeled S2, and mix (concentration of S2 = 2.5 ng/mL).
4. Pipette 150 µL of this dilution into the third tube, labeled S3, and mix thoroughly before the next transfer.
5. Repeat serial dilutions 4 more times thus creating the points of the standard curve (see Figure 7).

   Calibrator Diluent (1x) (for subsequent measurement of serum or plasma samples) or Sample Diluent (for subsequent measurement of cell culture supernatant samples) serves as blank.

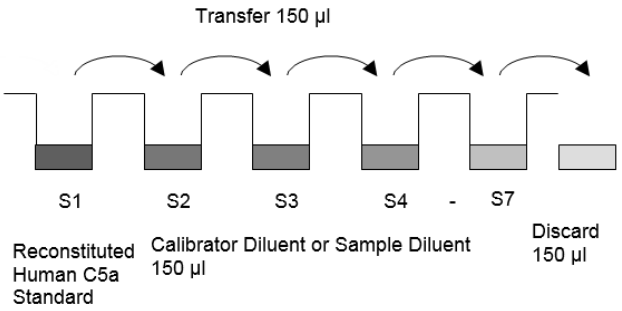

Fig. 7 External standard dilution

Test protocol

Note: For calculation only the predilution of the samples needs to be taken into account. For serum samples that have been prediluted 1:100, the dilution factor is 100. For plasma samples prediluted 1:25, the dilution factor is 25.

1. Predilute your serum plasma samples before starting with the test and dilute serum at least 1:100 and plasma 1:25 with Sample Diluent. For example :
   - Predilution serum: 1:100 (5 µL +495 µL Sample Diluent);
   - Predilution plasma (EDTA, Citrate, Heparin): 1:25 (10 µL prediluted sample + 240 µL Sample Diluent)
2. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2–8°C sealed tightly.

3. Wash the microwell strips twice with approximately 400 µL Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10–15 seconds before aspiration. Take care not to scratch the surface of the microwells.

   After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
4. Add 50 µL of Sample Diluent to all wells.
5. Add 50 µL of externally prepared standard dilution (see "External standard dilution" on page 3) in duplicate to designated standard wells according to Table 1.

   Table 1 Example of the arrangement of blanks, standards, and samples in the microwell strips.

	1	2	3	4
A	Standard 1 5.0 ng/mL	Standard 1 5.0 ng/mL	Sample 1	Sample 1
B	Standard 2 2.5 ng/mL	Standard 2 2.5 ng/mL	Sample 2	Sample 2
C	Standard 3 1.25 ng/mL	Standard 3 1.25 ng/mL	Sample 3	Sample 3
D	Standard 4 0.625 ng/mL	Standard 4 0.625 ng/mL	Sample 4	Sample 4
E	Standard 5 0.313 ng/mL	Standard 5 0.313 ng/mL	Sample 5	Sample 5
F	Standard 6 0.156 ng/mL	Standard 6 0.156 ng/mL	Sample 6	Sample 6
G	Standard 7 0.078 ng/mL	Standard 7 0.078 ng/mL	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

6. Add 50 µL of Calibrator Diluent (for serum or plasma samples) or Sample Diluent (for cell culture supernatant samples) in duplicate to the blank wells.
7. Add 50 µL of prediluted samples to all sample wells.
8. Cover with an adhesive film and incubate at room temperature (18–25°C) for 2 hours on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance).
9. Prepare Biotin-Conjugate (see "Biotin-Conjugate" on page 3).
10. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point 3 of the test protocol. Proceed immediately to the next step.
11. Add 100 µL of diluted Biotin-Conjugate to all wells, including the blank wells.
12. Cover with an adhesive film and incubate at room temperature (18–25°C) for 1 hour on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance.)
13. Prepare Streptavidin-HRP (see "Streptavidin-HRP" on page 3).
14. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point 3 of the test protocol. Proceed immediately to the next step.
15. Add 100 µL of diluted Streptavidin-HRP to all wells, including the blank wells.
16. Cover with an adhesive film and incubate at room temperature (18–25°C) for 1 hour on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance).
17. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point 3 of the test protocol. Proceed immediately to the next step.
18. Pipette 100 µL of TMB Substrate Solution to all wells.
19. Incubate the microwell strips at room temperature (18–25°C) for 30 minutes. Avoid direct exposure to intense light.

    The color development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay.

    It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9–0.95.
20. Stop the enzyme reaction by quickly pipetting 100 µL of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2–8°C in the dark.
21. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

Calculation of results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 percent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human C5a concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human C5a for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human C5a concentration.
- If instructions in this protocol have been followed, serum samples have been prediluted 1:100 and the concentration read from the standard curve must be multiplied by a dilution factor 100. Plasma samples have been prediluted 1:25 and the concentration read from the standard curve must be multiplied by a dilution factor 25.
- Calculation of samples with a concentration exceeding standard 1 require further external predilution according to expected human C5a values with Sample Diluent in order to precisely quantitate the actual human C5a level.
- It is suggested that each testing facility establishes a control sample of known human C5a concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.

- A representative standard curve is shown in Figure 8.

  Note: Do not use this standard curve to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

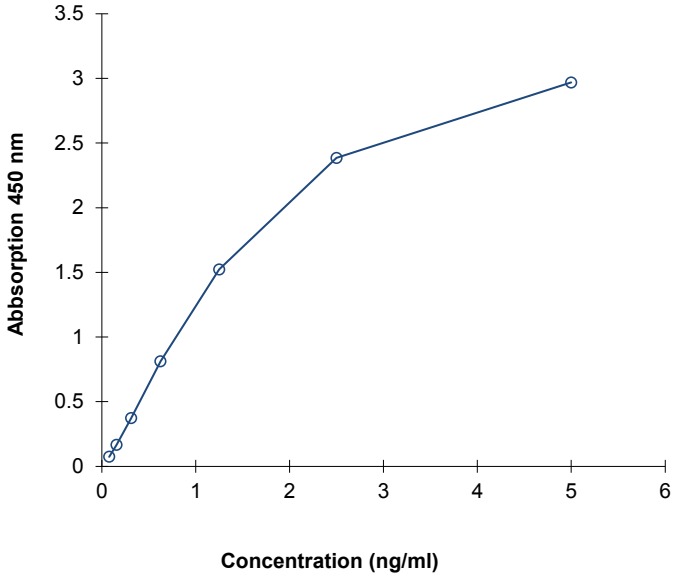

Fig. 8 Representative standard curve for human C5a ELISA. Human C5a was diluted in serial 2-fold steps in Calibrator Diluent.

Table 2 Typical data using the human C5a ELISA.

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	human C5a Concentration (ng/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	5.0	3.004 2.990	2.969	0.3
2	2.5	2.404 2.422	2.385	0.5
3	1.250	1.535 1.566	1.522	1.4
4	0.625	0.839 0.842	0.812	0.2
5	0.313	0.396 0.406	0.373	1.7
6	0.156	0.192 0.196	0.166	1.7
7	0.078	0.100 0.105	0.074	3.9
Blank	0.0	0.028 0.028	0.028	0

The OD values of the standard curve may vary according to the conditions of assay performance (e.g., operator, pipetting technique, washing technique, or temperature effects). Furthermore, shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Performance characteristics

Sensitivity

The limit of detection of human C5a defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.005 ng/mL (mean of 4 independent assays).

Reproducibility

Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 3replicates of 8 samples containing different concentrations of human C5a. Two standard curves were run on each plate. Data below show the mean human C5a concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 3.6%.

Table 3 The mean human C5a concentration and the coefficient of variation for each sample.

Sample	Experiment	Mean human C5a concentration (ng/mL)	Coefficient of variation (%)
1	1 2 3	7.0 6.7 6.9	3.6 2.6 4.4
2	1 2 3	11.4 10.9 11.0	2.5 2.2 5.4
3	1 2 3	11.5 10.5 11.6	2.0 1.8 3.5
4	1 2 3	5.6 5.0 5.4	2.9 2.4 3.8
5	1 2 3	4.2 4.0 4.0	4.3 7.4 9.3
6	1 2 3	1.1 1.1 1.1	3.6 4.6 3.9
7	1 2 3	0.7 0.6 0.7	3.8 3.4 2.1
8	1 2 3	0.3 0.3 0.3	3.2 2.8 2.1

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of human C5a. Two standard curves were run on each plate. Data below show the mean human C5a concentration and the coefficient of variation

calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 4.0%.

Table 4 The mean human C5a concentration and the coefficient of variation of each sample

Sample	Mean human C5a concentration (ng/mL)	Coefficient of variation (%)
1	6.85	1.8
2	11.09	2.1
3	11.23	5.4
4	5.32	5.9
5	4.03	3.1
6	1.10	1.8
7	0.67	6.1
8	0.28	5.7

Spike recovery

The spike recovery was evaluated by spiking 3 levels of human C5a into serum, plasma (EDTA, heparin, citrate), and cell culture supernatant. Recoveries were determined with 2 replicates each. The amount of endogenous human C5a in unspiked samples was subtracted from the spike values.

Sample matrix	Spike high (%)		Spike medium (%)		Spike low (%)	
	Mean	Range	Mean	Range	Mean	Range
Serum	100	95–117	79	74–83	88	73–102
Plasma (EDTA)	93	76–106	89	75–112	82	67–104
Plasma (citrate)	95	83–105	70	66–73	71	68–73
Plasma (heparin)	109	103–116	93	83–100	98	78–114
Cell culture supernatant	99	–	129		101	

Dilution parallelism

Serum, plasma (EDTA, citrate, heparin), and cell culture supernatant samples with different levels of human C5a were analyzed at serial 2-fold dilutions with 4 replicates each.

Sample matrix	Dilution	Recovery of exp. val. (%)	
		Mean	Range
Serum	1:100	102	81–123
	1:200	100	91–109
	1:400	100	92–110
Plasma (EDTA)	1:10	83	82–84
	1:20	92	65–117
	1:40	90	78–95
Plasma (citrate)	1:20	76	69–89
	1:40	83	70–96
	1:80	87	74–96
Plasma (heparin)	1:50	88	78–92
	1:100	101	89–109
	1:200	83	75–88
Cell culture supernatant	1:4	87	–
	1:8	101	–
	1:16	121	–

Specificity

The assay detects both natural and recombinant human C5a. The cross-reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human C5a positive sample. No cross-reactivity or interference was detected.

Reagent preparation summary

Wash buffer (1x)

Add Wash Buffer Concentrate 20x (50 mL) to 950 mL distilled water.

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

Assay buffer (1x)

Add Assay Buffer Concentrate 20x (5 mL) to 95 mL distilled water.

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

Biotin-Conjugate

Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

Streptavidin-HRP

Make a 1:200 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

Human C5a Standard

Reconstitute human C5a standard with Calibrator Diluent (serum or plasma samples) or Sample Diluent (cell culture supernatant samples). (Reconstitution volume is stated on the label of the standard vial.)

Test protocol summary

Note: For calculation only the predilution of the samples needs to be taken into account. For serum samples that have been prediluted 1:100, the dilution factor is 100. For plasma samples prediluted 1:25, the dilution factor is 25.

1. Predilute your samples before starting with the test procedure. (see "Test protocol" on page 3)
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.
4. Add 50 µL Sample Diluent to all wells.
5. External standard dilution in tubes (see "External standard dilution" on page 3): Pipette 50 µL of these standard dilutions in the microwell strips.
6. Add 50 µL of Calibrator Diluent (for serum or plasma samples) or Sample Diluent (for cell culture supernatant samples) in duplicate to the blank wells.
7. Add 50 µL of prediluted samples.
8. Cover microwell strips and incubate 2 hours at room temperature (18–25°C), on a microplate shaker (Shaking is absolutely necessary for an optimal test performance).
9. Prepare Biotin-Conjugate.
10. Empty and wash microwell strips 6 times with Wash Buffer.
11. Add 100 µL diluted Biotin-Conjugate to all wells.
12. Cover microwell strips and incubate 1 hour at room temperature (18–25°C), on a microplate shaker. (Shaking is absolutely necessary for an optimal test performance).
13. Prepare Streptavidin-HRP.
14. Empty and wash microwell strips 6 times with Wash Buffer.

15. Add 100 µL diluted Streptavidin-HRP to all wells.
16. Cover microwell strips and incubate 1hour at room temperature (18–25°C), on a microplate shaker (Shaking is absolutely necessary for an optimal test performance).
17. Empty and wash microwell strips 6 times with Wash Buffer.
18. Add 100 µL of TMB Substrate Solution to all wells.
19. Incubate the microwell strips for about 30 minutes at room temperature (18–25°C).
20. Add 100 µL Stop Solution to all wells.
21. Blank microwell reader and measure color intensity at 450 nm.

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