Human C5a (serum only)

HK349

Edition 06-24

ELISA KIT
PRODUCT INFORMATION & MANUAL

Read carefully prior to starting procedures!
For use in laboratory research only
Not for clinical or diagnostic use

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Please note that the user protocol provided is not specific to any particular lot and represents the general specifications for this product. We advise consulting the vial label and the Certificate of Analysis for information regarding specific lots. Additionally, be informed that shipping conditions for this product may differ from its recommended storage conditions.

This product is intended solely for research purposes and is not approved for human or animal use, or for diagnostic procedures. Users must adhere to all applicable local, state, and federal regulations when utilizing this product. Hycult Biotech disclaims any liability for patent infringements that may arise from the use or adaptation of this product.

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1. INTENDED USE

The human C5a ELISA kit is to be used for the *in vitro* quantitative determination of human C5a/C5a-desArg in serum only. The human C5a ELISA kit is not intended for measuring recombinant human C5a/C5adesArg. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

The analysis should be performed by trained laboratory professionals.

2. INTRODUCTION

C5a plays a pivotal role in the complement system, a key player in innate immunity. During the activation of complement protein 5 (C5), C5a, a potent anaphylatoxin comprising 74 amino acids, is produced. Initially short-lived, C5a is swiftly transformed in serum into the more stable C5a-desArg, maintaining biological activity and providing a reliable measure of complement activation levels in various samples.

C5a exerts profound physiological effects, inducing smooth muscle contraction, vasodilation, increased vascular permeability, and inflammatory mediator release from basophils and mast cells. It also serves as a chemotactic agent, drawing eosinophils, basophils, and monocytes to sites of inflammation.

The clinical relevance of C5a extends to inflammatory responses in conditions like gram-negative bacterial sepsis, trauma, ischemic heart disease, post-dialysis syndrome, and autoimmune diseases. Elevated C5a levels have been associated with increased cardiovascular risk and the capillary leak syndrome following bone marrow transplantation. Notably, C5a is a significant urinary biomarker for imminent acute graft rejection post-kidney transplantation.

Employing a specialized monoclonal antibody that recognizes a unique neo-epitope on C5a-desArg, the human C5a ELISA kit is designed for precision, eliminating the chance of cross-reactivity with C5. This precision positions the C5a ELISA kit as an essential tool for researchers delving into the complement system's intricacies.

3. KIT FEATURES

- Working time of 3½ hours.
- Minimum concentration which can be measured is 0.3 ng/ml.
- Measurable concentration range of 0.3 to 20 ng/ml.
- Working volume of 100 µl/well.

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Sample Type

Suitability of sample types that have been tested with human C5a ELISA:

Sampletype	Suitability
Plasma (EDTA/Heparin/Citrate)	No
Serum	Yes

Table 1

Other sample types have not been tested.

Cross reactivity

Potential cross-reacting proteins detected in the species human C5a ELISA:

Cross reactant	Reactivity
Rabbit, rat and pig C5a	Weak
Mouse C5a	No
Horse C5a	No
Human C3a	No

Table 2

Cross-reactivity for other species or proteins/peptides has not been tested.

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4. PROTOCOL OVERVIEW

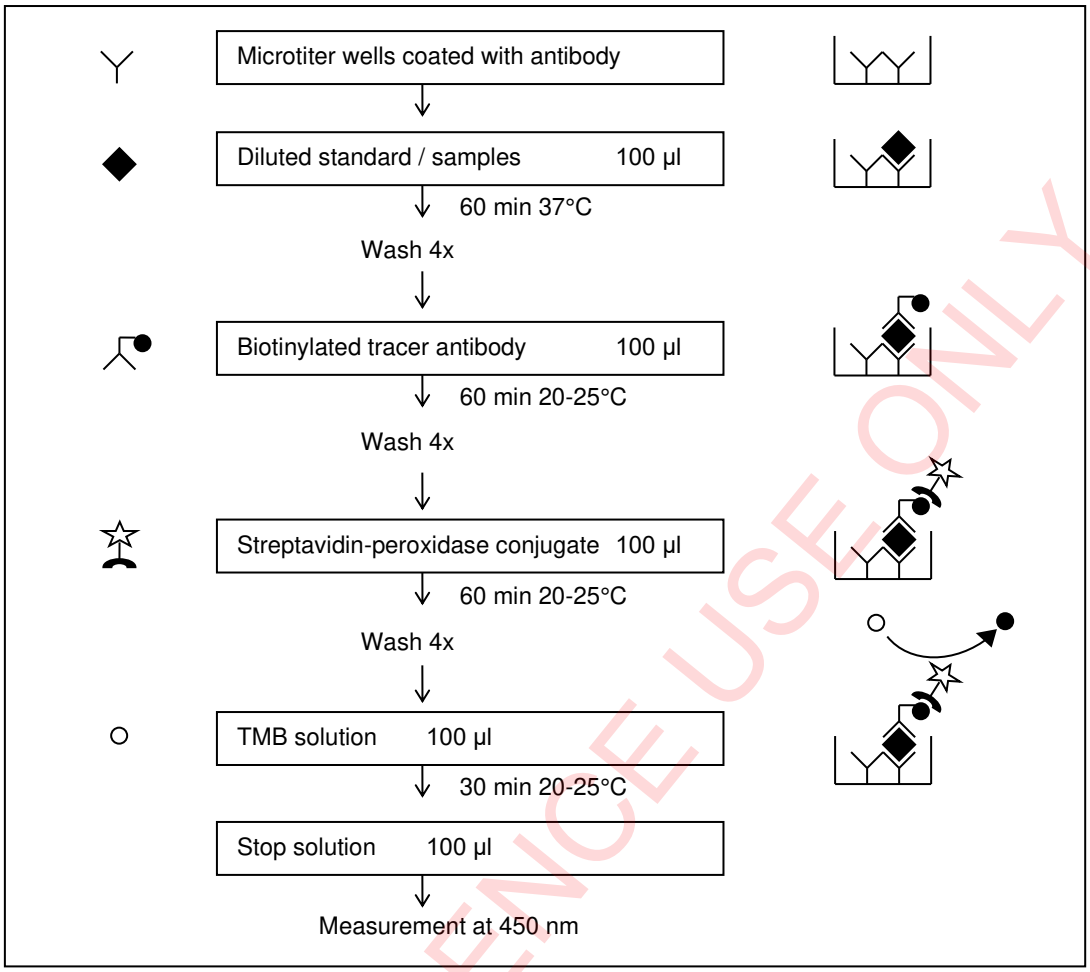

Figure 1

- The human C5a ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time of 3½ hours.
- The efficient format of a plate with twelve disposable 8-well strips allows free choice of batch size for the assay.
- Samples and standards are incubated in microtiter wells coated with antibodies recognizing human C5a.
- Biotinylated tracer antibody will bind to the captured human C5a.
- Streptavidin-peroxidase conjugate will bind to the biotinylated tracer antibody.
- Streptavidin-peroxidase conjugate will react with the substrate, tetramethylbenzidine (TMB).
- The enzyme reaction is stopped by the addition of oxalic acid.
- The absorbance at 450 nm is measured with a spectrophotometer. A standard curve is obtained by plotting the absorbance (linear) versus the corresponding concentrations of the human C5a standards (log).
- The human C5a concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.

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5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Kit component	Cat. #	Quantity HK349-01	Quantity HK349-02	Color code
Wash buffer 20x	WB21	1 vial (60 ml)	1 vial (60 ml)	Colorless
Dilution buffer 10x	DB81	1 vial (15 ml)	1 vial (15 ml)	Green
Standard		2 vials, lyophilized	4 vials, lyophilized	White
Tracer, biotinylated		1 vial, 1 ml lyophilized	2 vials, 1 ml lyophilized	White
Streptavidin-peroxidase 100x	CON03	1 tube, 0.25 ml in solution	1 tube, 0.25 ml in solution	Brown
TMB substrate	TMB050/TMB100	1 vial, (11 ml)	1 vial, (22 ml)	Brown
Stop solution	STOP110	1 vial, (22 ml)	1 vial, (22 ml)	Red
12 Microtiter strips, pre-coated		1 plate	2 plates	
Certificate of Analysis		1	1	
Manual		1	1	
Data collection sheet		2	2	

Table 3

- Store the kit components at 2 - 8°C immediately upon receipt. Do not freeze.
- Ensure components are used before the expiration date indicated on the kit label.
- The standard and tracer in lyophilized form and the streptavidin-peroxidase in concentrated solution are stable until the expiration date indicated on the kit label, if stored at 2 - 8°C.
- The exact quantity of the standard is specified on the vial label and the Certificate of Analysis.
- The standard is intended for a single use; after reconstitution, the standard remains stable for up to 12 hours. Stability beyond this duration is not assured, hence it cannot be stored after reconstitution.
- Once reconstituted the biotinylated tracer is stable for 1 month if stored at 2 - 8°C.
- Store the streptavidin-peroxidase exclusively in its concentrated form; it is not stable once diluted.
- Ensure that the foil pouch containing the plate is vacuum-sealed and undamaged upon receipt. Any deviation may impact assay performance.
- Immediately return unused strips back into the foil pouch with the desiccant, sealing it fully along the entire edge of the zip-seal. Stored at 2 - 8°C, the quality is ensured for one month.

Materials required but not provided

- Calibrated micropipettes and disposable tips.
- Distilled or de-ionized water.
- Plate washer: automatic or manual.
- Polypropylene tubes.
- Calibrated ELISA plate reader capable of measuring absorbance at 450 nm.
- Adhesive covers can be ordered separately. Please contact your local distributor.
- Centrifuge for 1 ml tubes.

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6. WARNINGS AND PRECAUTIONS

- This product is intended for research purposes only and not for use in diagnostic or therapeutic procedures.
- Only qualified personnel trained in laboratory procedures should handle this kit.
- Under no circumstances should sodium azide be added to any component as a preservative.
- Refrain from using kit components beyond their expiration date.
- To ensure accuracy, do not interchange reagents from different kits or lots. Each kit and lot is calibrated as a complete unit; use only the reagents supplied by the manufacturer.
- The assay is specifically optimized for the stated standard range. Alterations to the standard range are not recommended.
- Exercise caution when opening vials as they are under vacuum.
- Avoid ingestion of any kit components.
- The kit reagents include 2-chloroacetamide, a preservative known for its harmful effects upon skin contact and toxicity if ingested. In the event of an accident or discomfort, immediate medical consultation is advised.
- Protect the TMB substrate from intense light exposure; it should remain colourless until utilized.
- The stop solution contains 2% oxalic acid, a substance that can irritate or burn the respiratory system, skin, and eyes. Avoid any direct contact, and in case of exposure, rinse thoroughly with water and seek medical attention.
- Deviations from the specified incubation times, temperatures, or pipetting volumes may result in inaccurate results.
- Once dispensed, avoid reusing microwells or returning reagents to their original bottles.
- Treat all biological samples as potentially hazardous or infectious and handle them under conditions that minimize the risk of disease transmission.
- Be aware that samples that are hemolyzed, hyperlipemic, heat-treated, or contaminated may yield inaccurate results.
- Utilize polypropylene tubes for the preparation of standards and samples, avoiding the use of polystyrene tubes or sample plates.
- The standard is derived from human sources and has been tested for various viruses with negative results. However, as no testing method can guarantee the complete absence of infectious agents, treat this reagent with the same precautions as you would any potentially infectious human serum or blood specimen. Follow established guidelines for preventing the transmission

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7. SAMPLE PREPERATION

Collection and handling

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 serum (immediately after clotting).

Serum

Allow freshly collected blood to clot by standing tubes vertically at room temperature for 60 min. Centrifuge the clotted blood (1500xg at 4°C for 15 min). Transfer the serum to a fresh polypropylene tube.

Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Storage at -20°C can affect recovery of human C5a. Use samples within 24 hours after thawing. Avoid multiple freeze-thaw cycles which may cause loss of human C5a activity and give erroneous results.

Do not use hemolyzed, hyperlipemic, heat-treated or contaminated samples.

Before performing the assay, samples should be brought to room temperature (18 – 25°C) and mixed gently. Prepare all samples (controls and test samples) prior to starting the assay procedure. Avoid foaming.

Dilution procedures

Serum samples

Human C5a can be measured accurately if serum samples are diluted at least 4x with supplied dilution buffer in polypropylene tubes. For activated serum human C5a can be measured accurately if serum samples are diluted at least 100x.

Comment regarding recommended sample dilution

The mentioned dilution for samples is a minimum dilution and should be used as a guideline. The recovery of human C5a from an undiluted sample is not 100% and may vary from sample to sample. When testing less diluted samples it is advisable to run recovery experiments to determine the influence of the matrix on the detection of human C5a.

Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.

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Guideline for dilution of samples

Please see the table below for recommended sample dilutions. Volumes are based on a total volume of at least 230 µl of diluted sample, which is sufficient for one sample in duplicate in the ELISA. For dilution of samples we recommend to use at least 10 µl of sample.

	Dilution	Pre-dilution	Amount of sample or pre-dilution required	Amount of Dilution buffer required
1.	10x	Not necessary	25 µl (sample)	225 µl
2.	20x	Not necessary	15 µl (sample)	285 µl
3.	50x	Not necessary	10 µl (sample)	490 µl
4.	100x	Not necessary	10 µl (sample)	990 µl
5.	500x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	490 µl
6.	1000x	Recommended: 10x (see nr.1)	10 µl (pre-dilution)	990 µl
7.	2000x	Recommended: 20x (see nr.2)	10 µl (pre-dilution)	990 µl
8.	5000x	Recommended: 50x (see nr.3)	10 µl (pre-dilution)	990 µl

Table 4

8. REAGENT PREPARATION

Allow all the reagents to equilibrate to room temperature (20 – 25°C) prior to use. Return to proper storage conditions immediately after use.

Wash buffer

Prepare wash buffer by mixing 60 ml of 20x wash buffer with 1140 ml of distilled or de-ionized water, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of wash buffer by diluting 1 part of the 20x wash buffer with 19 parts of distilled or de-ionized water.

Dilution buffer

Prepare dilution buffer by mixing 15 ml of the 10x dilution buffer with 135 ml of distilled or de-ionized water, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of dilution buffer by diluting 1 part of the 10x dilution buffer with 9 parts of distilled or de-ionized water. Concentrated dilution buffer may contain crystals. In case the crystals do not disappear at room temperature within 1 hour, concentrated dilution buffer can be warmed up to 37°C. Do not shake the solution.

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Standard solution

The standard is reconstituted by pipetting the amount of dilution buffer mentioned on the CoA in the standard vial. Use the standard vial as Tube 1 in Figure 2. Prepare each C5a standard in polypropylene tubes by serial dilution of the reconstituted standard with dilution buffer as shown in Figure 2*. After reconstitution the standard must be used within 12 hours and cannot be stored for repeated use.

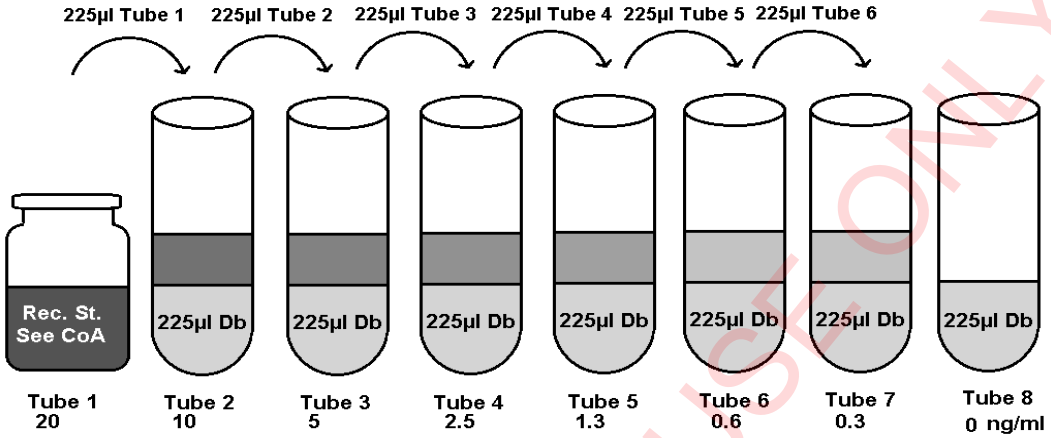

Figure 2

*)CoA: Certificate of Analysis, Rec. St.: Reconstituted Standard, Db: Dilution buffer

Tracer solution

The tracer is reconstituted by pipetting of 1 ml distilled or de-ionized water. Dilute the reconstituted 1 ml tracer with 11 ml dilution buffer, which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of tracer by diluting 1 part of the reconstituted tracer with 11 parts of dilution buffer.

Streptavidin-peroxidase solution

It is advised to spin down streptavidin-peroxidase tubes before use. Prepare the streptavidin-peroxidase solution by mixing 0.25 ml of the 100x streptavidin-peroxidase solution with 24.75 ml dilution buffer, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of streptavidin-peroxidase solution by diluting 1 part of the 100x streptavidin-peroxidase solution with 99 parts of dilution buffer.

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9. ELISA PROTOCOL

Bring all reagents to room temperature (20 - 25°C) before use.

1. Determine the number of test wells required, put the necessary microwell strips into the supplied frame, and fill out the data collection sheet. Return the unused strips to the storage bag with desiccant, seal and store at 2 - 8°C.
   Please notice step 2 followed by placing the plate at 37°C should be performed within 15 minutes.
2. Transfer within 100 µl in duplicate of standard, samples, or controls into appropriate wells. Do not touch the side or bottom of the wells.
3. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
4. Incubate the strips or plate for 1 hour at 37°C.
5. Wash the plates 4 times with wash buffer using a plate washer or as follows*:
   a. Remove the cover, avoid splashing.
   b. Empty the plate by inverting plate and shaking contents out over the sink, keep inverted and tap dry on a thick layer of tissues.
   c. Add 200 µl of wash buffer to each well, wait 20 seconds, empty the plate as described in 5b.
   d. Repeat the washing procedure 5b/5c three times.
   e. Empty the plate and gently tap on thick layer of tissues.
6. Add 100 µl of diluted tracer to each well using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
7. Cover the tray and incubate the tray for 1 hour at room temperature.
8. Repeat the wash procedure described in step 5.
9. Add 100 µl of diluted streptavidin-peroxidase to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
10. Cover the tray and incubate the tray for 1 hour at room temperature.
11. Repeat the wash procedure described in step 5.
12. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
13. Cover the tray and incubate the tray for 30 minutes at room temperature. It is advised to control the reaction on the plate regularly. In case of strong development the TMB reaction can be stopped sooner. Avoid exposing the microwell strips to direct sunlight. Covering the plate with aluminum foil is recommended.
14. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 12. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any air bubbles trapped in the wells.
15. Read the plate within 30 minutes after addition of stop solution at 450 nm using a plate reader, following the instructions provided by the instrument's manufacturer.

*) In case plate washer is used, please note: use of a plate washer can result in higher background and decrease in sensitivity. We advise validation of the plate washer with the manual procedure.
Make sure the plate washer is used as specified for the manual method.

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10. INTERPRETATION OF RESULTS

- Determine the average absorbance for each group of duplicate standards, controls, and samples.
- Discrepancies exceeding 15% from the mean absorbance value suggest potential inaccuracies, necessitating sample reanalysis.
- Ensure the mean absorbance of the zero standard does not surpass 0.3.
- Utilize specialized software to construct a standard curve, plotting mean absorbance values (Y-axis) against corresponding concentrations (X-axis) on a logarithmic scale.
- For diluted samples, adjust the concentration derived from the standard curve by the dilution factor.
- Samples yielding an average absorbance higher than that of the maximum standard concentration exceed the assay's scope and must be reanalysed using a greater dilution factor.

11. TECHNICAL HINTS

- Technicians should be proficient and well-versed in ELISA assays and the specific test procedures before initiating the assay.
- For those unfamiliar with ELISA techniques, it is advisable to conduct a preliminary assay with a standard curve to ensure understanding and adherence to the protocol before proceeding with sample evaluations.
- Accurate and thorough washing is critical at all stages of the assay to prevent false positive or negative outcomes. Ensure complete removal of liquids from wells prior to adding wash buffer, adhere strictly to the specified volume for each washing cycle, and avoid allowing the wells to remain uncovered or dry for prolonged periods.
- A standard curve is essential for each assay run due to varying conditions; samples must be evaluated against a standard curve established on the same plate during that session.
- Do not interchange reagents from different kits or batches, including strips, and avoid combining remnants with contents from new vials.
- Prepare fresh dilutions of the standard, samples, biotinylated LPS, streptavidin-peroxidase, and buffers each time the kit is utilized.
- Maintain cap-to-vial correspondence; caps are designed to fit their original vials and should not be swapped.
- Prevent cross-contamination by using new pipette tips for each addition across standards, samples, and reagents, and employ separate reservoirs for each reagent to ensure integrity.
- Dispose of all waste in accordance with the established laboratory safety protocols and regulations.

Technical support

For any questions or technical support related to the ELISA, please feel free to reach out to our technical support team at support@hycultbiotech.com.

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12. QUALITY CONTROL

The Certificate of Analysis accompanying this kit is specific to its lot number, intended to verify the results achieved in your facility. Please note that the absorbance values indicated on the Certificate serve merely as reference points; deviations in outcomes produced by your laboratory are to be expected. Designed to mitigate the impact of soluble receptors, binding proteins, and extraneous variables present in biological samples, this assay aims for precise measurement free from external interferences. Nonetheless, without exhaustive testing of all possible variables, the exclusion of interference cannot be entirely assured.

To ensure the highest efficacy of this kit, implementing good laboratory practices(GLP) is crucial.

13. PERFORMANCE CHARACTERISTICS

Linearity

The linearity of the assay was determined by serially diluting a sample containing 331 ng/ml human C5a. The diluted samples were measured in the assay. The line obtained a slope of 1.0907 and a correlation coefficient of 0.9988

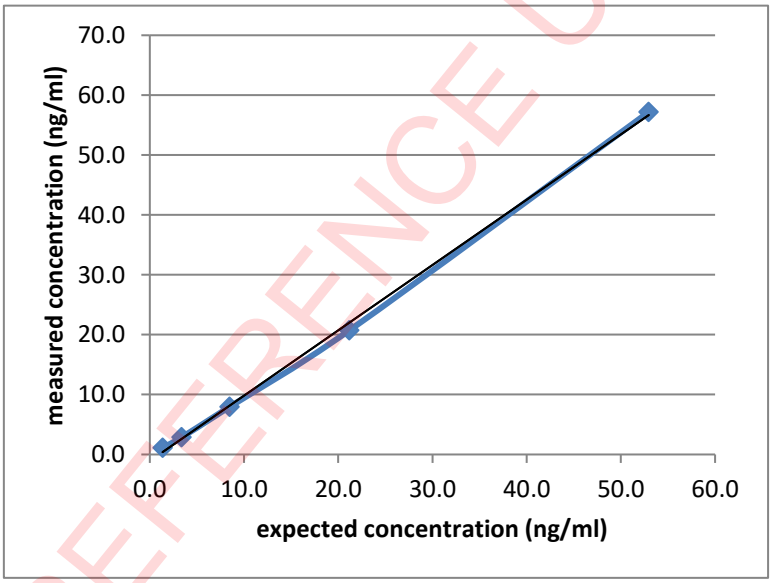

Figure 3

Recovery

Serum

Normal human serum containing baseline levels of human C5a, were spiked with human C5a, in concentrations of 10 and 2.5 ng/ml. Samples with and without human C5a, were incubated for 1 hour at 37°C. Samples were measured using the ELISA. Recovery values for human C5a, ranged between 89% and 102% .

Activated serum

Normal activated human serum containing baseline levels of human C5a, were spiked with human C5a, in concentrations of 10 and 2.5 ng/ml. Samples with and without human C5a,

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were incubated for 1 hour at 37°C. Samples were measured using the ELISA. Recovery values for human C5a, ranged between 99% and 108% .

14. TROUBLESHOOTING

Warranty claims and complaints in respect of deficiencies must be logged before expiry date of the product. A written complaint containing lot number of the product and experimental data shall be sent to support@hycultbiotech.com.

Suggestions summarized below in Table 4 can be used as guideline in case of unexpected assay results.

Low absorbance	High absorbance	Poor duplicates	All wells positive	All wells negative	Possible cause
•	•		•	•	Kit materials or reagents are contaminated or expired
•					Incorrect reagents used
•		•	•		Lyophilized reagents are not properly reconstituted
•	•	•	•	•	Incorrect dilutions or pipetting errors
•		•			Improper plastics used for preparation of standard and/or samples
•	•				Improper incubation times or temperature
		•			Especially in case of 37°C incubation: plates are not incubated uniformly
•					Assay performed before reagents had reached room temperature
•	•	•	•	•	Procedure not followed correctly
				•	Omission of a reagent or a step
		•			Poor mixing of samples
	•		•		Low purity of water
	•	•			Strips were kept dry for too long during/after washing
	•	•	•		Inefficient washing
	•	•			Cross-contamination from other samples or positive control
		•	•		TMB solution is not clear or colorless
•	•				Wrong filter in the microtiter reader
	•	•			Airbubbles
		•			Imprecise sealing of the plate after use
•					Wrong storage conditions
•					Lamp in microplate reader is not functioning optimally

Table 5

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15. REFERENCES

1. Morad H et al; Time-course analysis of C3a and C5a quantifies the coupling between the upper and terminal Complement pathways in vitro. J Immunol Meth 2015, *427:* 13
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3. Stove S et al: Circulating complement proteins in patients with sepsis or systemic inflammatory response syndrome. Clin Diag Lab Immunol 1996, *3*:175
4. Krug N et al; Complement factors C3a and C5a are increased in bronchoalveolar lavage fluid after segmental allergen provocation in subjects with asthma. Am J Respir Crit Care Med 2001, *164*:1841

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