

## C5a ELISA

**Cat.-No. : IB79153**

**Size : 24 x 8**  
**(Break apart)**

Enzyme immunoassay (microtiter strips) for the quantitative  
determination of anaphylatoxin C5a  
in human plasma and urine

**Storage: 2-8°C**

Regulatory Status EU: For in-vitro diagnostic use only  
USA: Research use only

May 2004

Version 3.0  
Effective May 2004

## 1. INTRODUCTION

The **C5a** Enzyme Immunoassay Kit provides materials for the quantitative determination of Anaphylatoxin C5a in human plasma or urine.

The complement system consists of more than 20 proteins which evolved as defense system against invading microorganisms. It can also be activated in a variety of disease states or upon contact with medical devices or drugs (1). Upon activation, a cascade of proteolytic enzymes releases the anaphylatoxins C3a, C4a and C5a from their respective precursors (2). These fragments exert various biological functions such as histamine release, smooth muscle contraction, increase in capillary permeability or immunomodulation (3). In addition, C5a and its degraded form C5a-desArg are highly potent chemotactic agents for polymorphonuclear leukocytes, which then will release tissue degradative enzymes and oxygen radicals (4). This in turn will also lead to activation of other humoral systems such as coagulation and fibrinolysis (5). Thus, C5a is probably the most important complement-derived proinflammatory mediator. C5a is believed to play a pivotal role in the pathogenesis of septic shock, the adult respiratory distress syndrome, acute pancreatitis and the deleterious effects after myocardial infarction (6,7,8). Recently it has been shown that C5a is closely associated with the capillary leak syndrome in leukemic children after bone marrow transplantation. C5a is also a marker in urine for predicting the onset of acute graft rejection after kidney transplantation (9). With respect to possible deleterious consequences, C5a determination may be indicated during hemodialysis, after cardiopulmonary bypass or after any other contact with medical devices (10).

## 2. PRINCIPLE OF THE TEST

The **C5a ELISA KIT** is an enzyme immunoassay for the in vitro determination of human anaphylatoxin C5a and is based on the sandwich principle.

Due to cross-reactivity of the monoclonal antibodies with complement factor C5, C5 in the sample is removed by precipitation prior to analysis. The resulting clear supernatant contains the C5a to be determined (10,11). During the first incubation the C5a in the sample binds to murine anti C5a monoclonal antibodies (mab 561), which are attached to the surface of the microtitration plate. Unbound constituents are then removed by washing and, in a second reaction, peroxidase conjugated monoclonal antibodies (Mab 557) are added and bound to a different epitope on C5a. The excess enzyme conjugated antibodies are removed by washing; the bound enzyme activity is then determined. The enzymatic reaction between hydrogen peroxide and chromogen is terminated by the addition of dilute sulphuric acid. The intensity of the colour intensity, which is proportional to the concentration of C5a, is determined photometrically. The concentration range of approx. 0.1 to 10 µg/L (the exact values are indicated on the labels) is covered by the standards contained in the kit. For higher concentrations the sample must be diluted with dilution reagent.

## 3. PRECAUTIONS

- Not for in-vitro diagnostic use! For research purposes only!
- For information on hazardous substances included in the kit please refer to Material Safety Data Sheets.
- All reagents of this test kit which contain human serum or plasma have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
- Avoid contact with Stop Solution containing 0.5 M H<sub>2</sub>SO<sub>4</sub>. It may cause skin irritation and burns.
- Never pipet by mouth and avoid contact of reagents and specimens with skin and mucous membranes.
- Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
- Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
- Handling should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.
- Do not use reagents beyond expiry date as shown on the kit labels.
- All indicated volumes have to be performed according to the protocol. Optimal test results are only obtained when using calibrated pipettes.
- Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even if the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may result slightly different.
- Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guideline or regulation.
- Safety Data Sheets for this product are available upon request.  
The Safety Data Sheets fit the demands of: EU-Guideline 91/155 EC.

## 4. KIT COMPONENTS

### 4.1. Contents of the Kit

1. **Microtiterwells**, 24x8 (break apart) strips, 192 wells  
Wells coated with murine monoclonal antibodies against human C5a
2. **Standard (Standard 1-4)**, 4 vials (lyoph.), 1.0 ml  
0.1; 0.4, 3.0 and 10.0 µg/l  
see „Preparation of Reagents“
3. **Control**, 1 vial (lyoph.), 1.0 ml  
see „Preparation of Reagents“
4. **Assay Buffer**, 1 vial, 25 ml, ready to use
5. **Enzyme Conjugate**, 1 vial, 0.5 ml, concentrate  
Murine monoclonal antibodies to human C5a, conjugated to horseradish peroxidase  
see „Preparation of Reagents“
6. **Conjugate Diluent**, 2 vials, 11 ml each, ready to use  
Tris Buffer solution (50 mmol/L)
7. **Precipitation Reagent**, 1 vial, 20 ml, ready to use
8. **Substrate Solution**, 1 vial, 25ml, ready to use  
TMB
9. **Stop Solution**, 1 vial, 25ml, ready to use  
contains 0.5M H<sub>2</sub>SO<sub>4</sub>  
Avoid contact with the stop solution. It may cause skin irritations and burns.
10. **Wash Solution**, 1 vial, 30 ml (40X concentrated)  
see „Preparation of Reagents“

#### 4.1.1 Equipment and material required but not provided

1. Tris Buffer Solution (Tris/HCl Buffer pH 8.0 Tris (100 mmol/L), NaCl (25 mmol/L). For sample dilution. (This solution can be ordered.)
2. Centrifuge: suited for small reaction tubes (e.g. Eppendorf).
3. A microtiterplate calibrated reader (450±10 nm).
4. Calibrated variable precision micropipettes.
5. Absorbent paper.
6. Aqua dest.

#### 4.2 Storage and stability of the Kit

- When stored at 2° to 8°C unopened reagents will retain reactivity until expiration date. Do not use reagents beyond this date.
- Enzyme-Conjugate, Substrate Solution, Standards and Control must be stored at 2° to 8°C.
- Microtiter wells must be stored at 2° to 8°C. Once the foilbag has been opened, care should be taken to close it tightly again.
- Enzyme Conjugate, Conjugate Diluent, Assay Buffer and Precipitation Reagent may be used within 4 weeks after opening.
- The Precipitation Reagent has to be stored protected from light.

#### 4.3 Preparation of Reagents

Allow all reagents and required number of strips to reach room temperature prior to use.

#### Standards and Control

Reconstitute the lyophilized contents of the standard and control vial with 1.0 ml Aqua dest.

**Note:** The reconstituted standards and control can be used within 8 hours at +15 to +25°C or within 1 day at +2 to 8°C. For longer storage freeze at -20°C for 4 weeks. Frozen (-20°C) reconstituted Standards or Control should only be used once within 4 weeks.

#### Working Conjugate Solution

Pipette 200 µl of Anti-human C5a Conjugate into a vial of Conjugate Diluent (11 ml) and shake gently to mix (sufficient for 1 test plate).

Working Conjugate Solution can be stored at +2 to +8°C for 4 weeks.

#### Wash Solution

Dilute 30 ml of concentrated Wash Solution with 1170 ml deionized water to a final volume of 1200 ml. The diluted Wash Solution is stable for 2 weeks at room temperature.

**Dilution Reagent** (for Sample dilution)

Prepare a Dilution Reagent by mixing an equal volume of Tris buffer with distilled water.

**4.4 Disposal of the Kit**

The disposal of the kit must be made according to the national official regulations. Special information for this product are given in the Material Safety Data Sheets (see chapter 13).

**4.5 Damaged Test Kits**

In case of any severe damage of the test kit or components, IBL-Hamburg has to be informed written, latest one week after receiving the kit. Severely damaged single components should not be used for a test run. They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

**5. SPECIMEN****5.1 Specimen collection****Plasma**

Plasma is to be collected in appropriate tubes containing EDTA as anticoagulant. Centrifuge within 2 hours for 10 min. at a minimum of 1500 x g and remove the supernatant plasma.

C5a is preferentially determined in plasma or urine stabilized with EDTA ( $\geq 10$  mmol/L final concentration). Citrated plasma may also be used but requires special care as e.g. immediate cooling at ice in order to avoid unspecific activation of the complement cascade (13).

Haemolytic and lipaemic plasma and plasma containing rheumatoid factors do not interfere with the assay.

**Urine**

In urine C5a is stable at room temperature (+15 to +25°C) for 24 hours (9). Thus, urine routinely collected over 24 hours can be used as well as spontaneous urine. In case of severe proteinuria additional cleavage of excreted C5 might occur.

For collection of urine 1 part of an appropriate EDTA solution ( $>0.11$  nmol/L) is mixed with 9 parts of urine.

**5.2 Specimen storage**

Stability of <b>plasma</b> sample:	+15 to 25°C	2 hours
	+2 to +8°C	8 hours
	-20°C	1 month

Thawed samples should be inverted several times prior to testing.

Stability of <b>urine</b> sample:	+15 to + 25°C	24 hours
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**5.3 Specimen dilution**

If high values are expected dilute the plasma sample first 1:10 with Dilution Reagent and then apply the normal precipitation step to the diluted sample.

In the case that the amount of sample is limited the further dilution can be prepared from the supernatant. But for exact results, the sample dilution must be done before the precipitation step.

**5.4 Preparation of Samples - Precipitation**

In order to exclude cross-reactivity of the monoclonal antibodies with uncleaved complement factor C5, the C5 in standards, control plasma and samples has to be removed by precipitation. After centrifugation the clear supernatant contains the C5a to be analysed.

1. Pipette into appropriate centrifugation tubes one volume of either sample, standard or control plasma and add one volume of the Precipitation Reagent. For double determinations a volume of 100  $\mu$ l of sample and 100  $\mu$ l of Precipitation Reagent is recommended.
2. Mix intensively at once and incubate at least for 3 min. at +15 to +25°C.
3. Centrifuge the mixture for 10 min. at approx. 2500 x g (or 3 min. at 8000 x g).
4. **Use the clear supernatant in the assay procedure.**

In the supernatant C5a is stable at +15 to 25°C for 1 day and at +2 to +8°C for 3 days if stored separately from the pellet.

## 6. TEST PROCEDURE

### 6.1 General Remarks

- All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming.
- Once the test has been started, all steps should be completed without interruption.
- Use new disposal plastic pipet tips for each standard, control of sample in order to avoid crosscontamination
- Absorbance is a function of the incubation time and temperature. Before starting the assay, it is recommended that all reagents be ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.
- The present kit is adjusted to give an absorption for the highest standard > 1.200 within 10 minutes at room temperature. As a general rule the enzymatic reaction is linearly proportional to time and temperature. Therefore, if the Optical Density is too high or too low, the substrate incubation time can be decreased or increased, respectively.

### 6.2 Procedural Notes

- All standards, samples, and controls should be run in duplicate concurrently so that all conditions of testing are the same.
- The concentration of the samples can be read directly from this standard curve. Samples with a concentration higher than that of the highest standard have to be diluted 1 : 10 with Dilution Reagent. For the calculation of the concentrations this dilution factor has to be taken into account.

### 6.3 Assay Procedure

#### 6.3.1 Assay Procedure for Plasma Samples

1. Secure the desired number of Microtiterwells in the holder.
  2. Pipette into each well **50 µl** of Assay Buffer (C5a).
  3. Dispense **50 µl** of the supernatant of either standard, control or sample **with new disposable tips** into appropriate wells. After filling the test plate shake briefly to ensure thorough mixing.
  4. Incubate for **20 min.** ( ± 2 min.) at room temperature (+20 to +25°C).
  5. Briskly shake out the contents of the wells.  
Rinse the wells 3 times with diluted Wash Solution (300 µl per well). Strike the wells sharply on absorbent paper to remove residual water droplets.
- Important note:**  
The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!
6. Dispense **100 µl** Working Conjugate Solution into each well.
  7. Incubate for **15 min.** ( ± 2 min.) at room temperature (+20 to +25°C).
  8. Briskly shake out the contents of the wells.  
Rinse the wells 3 times with diluted Wash Solution (300 µl per well). Strike the wells sharply on absorbent paper to remove residual water droplets. (See step 5.)
  9. Add **100 µl** of Substrate Solution to each well.
  10. Incubate for **15 minutes** ( ± 2 min.) at room temperature.
  11. Stop the enzymatic reaction by adding **100 µl** of Stop Solution to each well.
  12. Read the OD at **450±10 nm** with a microtiterplate reader **immediately** after adding the Stop Solution.

#### 6.3.2 Assay Procedure for Urine Samples

For testing urine samples the following procedure is recommended to obtain an improved recovery of C5a.

1. Instead of using Standards S1 to S4 for establishing the reference curve **use only standard S4** and prepare a precipitate as described.
2. Then dilute the supernatant in series (1:2, 1:4, 1:8, 1:16) with a 1:1 mixture of the Precipitation Reagent and the following buffer:  
150 mmol/Na-phosphate, 150 mmol/L NaCl, 10 mmol/L EDTA, pH 7.0.
3. For dilution of urine samples follow the same procedure, i.e. dilute the supernatant after precipitation with the phosphate buffered saline/Precipitation Reagent mixture.
4. With this prepared Standard curve and urine samples follow now the procedure as described in "6.3.1 Assay Procedure for Plasma Samples".

## 6.4 Calculation of Results

1. Calculate the average absorbance values for each set of standards, controls and patient samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical(Y) axis and concentration on the horizontal (X) axis on log-log graph paper.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve. Depending on experience and/or the availability of computer capability, other methods of data reduction may be employed.
4. Automated method: Computer programs using 4 PL (4 Parameter Logistics) or Logit-Log can generally give a good fit.
5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be diluted with Dilution Reagent. For the calculation of the concentrations this dilution factor has to be taken into account.

## 7. ASSAY CHARACTERISTICS

### 7.1 Expected values

It is strongly recommended that each laboratory should determine its own normal and abnormal values.

#### Preliminary Reference Interval:

The Normal Values of the ELISA were determined by measuring the Values of 240 apparently healthy adults with the ELISA Kit.

The normal value range is assumed to be as 2.5th – 97.5th percentile.

	Median µg/L	Range µg/L
Adults	0.35	0.15 – 0.5 µg/L

### 7.2 Specificity

Data can be obtained on request.

### 7.3 Sensitivity

The minimum detectable concentration of C5a by this assay is estimated to be < 0.02 µg/l

### 7.4 Accuracy

#### Quality Control

It is recommended to use control samples according to state and federal regulations. The use of control samples is advised to assure the day to day validity of results. Use controls at both normal and pathological levels.

The controls and the corresponding results of the QC-Laboratory are stated in the QC certificate added to the kit. The values stated on the QC sheet always refer to the current kit lot and should be used for direct comparison of the results.

It is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results.

Employ appropriate statistical methods for analysing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid.

In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods.

After checking the above mentioned items without finding any error contact your distributor or IBL directly.

### 7.5 Precision

#### 7.5.1 Intra Assay Variation

In the range between 2 and 3 µg/L the coefficient of variation in the series (intra-assay CV) was found between 5 and 8%.

### **7.5.2 Inter Assay Variation**

In the range between 2 and 3 µg/L the coefficient of variation from day to day (inter-assay CV) was found between 6 and 10%.

### **7.6 Recovery**

The recovery of C5a in plasma was between 86 and 114%.

### **7.7 Linearity**

Data can be obtained on request.

## **8. LIMITATIONS OF USE**

### **8.1 Interfering Substances**

Any improper handling of samples or modification of this test might influence the results. Interferences caused by improper sample handling are explained in the chapters 'Specimen - Collection'.

**Note: Incorrect collection technique, e.g. inadequate mixing of the sample and anti-coagulant, can lead to falsely elevated C5a values!**

### **8.2 High-Dose-Hook Effect**

Data can be obtained on request.

## **9. LEGAL ASPECTS**

### **9.1 Reliability of Results**

The test must be performed exactly as per the manufacturer's instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.

The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact IBL.

### **9.2 Therapeutical Consequences**

Therapeutical consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 9.1. Any laboratory result is only a part of the total clinical picture of a patient.

Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutical consequences be derived.

The test result itself should never be the sole determinant for deriving any therapeutical consequences.

### **9.3 Liability**

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement.

Claims submitted due to customer misinterpretation of laboratory results subject to point 9.2. are also invalid. Regardless, in the event of any claim, the manufacturer's liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.

## 10. REFERENCES

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