C5a ELISA Kit

Enzyme Immunoassay for the quantification of C5a in human plasma and urine samples. (This kit also cross-react to C5a from rabbit, pig and rhesus monkey samples.)

Catalog number: ARG80868

Package: 2 x 96 wells

For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

SECTION	Page
INTRODUCTION	3
PRINCIPLE OF THE ASSAY	3
MATERIALS PROVIDED & STORAGE INFORMATION	4
MATERIALS REQUIRED BUT NOT PROVIDED	5
TECHNICAL HINTS AND PRECAUTIONS	5
SAMPLE COLLECTION & STORAGE INFORMATION	6
REAGENT PREPARATION	8
SAMPLE PRECIPITATION	9
ASSAY PROCEDURE	9
CALCULATION OF RESULTS	11
EXAMPLE OF TYPICAL STANDARD CURVE	12
QUALITY ASSURANCE	12

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INTRODUCTION

Activation of C5 by a C5 convertase initiates the spontaneous assembly of the late complement components, C5-C9, into the membrane attack complex. C5b has a transient binding site for C6. The C5b-C6 complex is the foundation upon which the lytic complex is assembled.

Derived from proteolytic degradation of complement C5, C5 anaphylatoxin is a mediator of local inflammatory process. Binding to the receptor C5AR1 induces a variety of responses including intracellular calcium release, contraction of smooth muscle, increased vascular permeability, and histamine release from mast cells and basophilic leukocytes. C5a is also a potent chemokine which stimulates the locomotion of polymorphonuclear leukocytes and directs their migration toward sites of inflammation. [UniProt]

PRINCIPLE OF THE ASSAY

This is an Enzyme Immunoassay for the quantification of C5a in plasma and urine samples.

This assay employs the sandwich enzyme immunoassay technique for the detection of C5a in human, rabbit, pig and rhesus monkey plasma and urine.

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 only C5a should be determined. 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 microtiter plate.

Unbound constituents are then removed by washing. 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 acidic solution. The concentration of C5a in test sample is directly proportional to the color intensity, which can be determined by extrapolation to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8°C. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated microplate	24 X 8 strips	4°C
Standard 1-4 (0.1, 0.4, 3.0, 10.0 µg/L)	4 vials (Lyophilized)	4°C
Control (2.41 µg/L; acc. range: 1.65-3.07 µg/L)	1 vial (Lyophilized)	4°C
Assay Buffer	25 ml (Ready-to-use)	4°C
HRP-conjugated C5a antibody concentrate	0.5 ml	4°C
Antibody Diluent	2 X 11 ml (Ready-to-use)	4°C
Precipitation Reagent	20 ml (Ready-to-use)	4°C (Protect from light)
40X Wash Solution	30 ml	4°C
TMB substrate	25 ml (Ready-to-use)	4°C (Protect from light)
STOP solution	25 ml (Ready-to-use)	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Tris buffer solution (Tris/HCl Buffer pH 8.0 Tris (100 mmol/L), NaCl (25 mmol/L). For sample dilution.
- Pipettes and pipette tips
- Deionized or distilled water
- Automated microplate washer (optional)
- Orbital shaker

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 4°C at all times. Microtiter wells must be stored at 2 - 8°C. Once the foil bag has been opened, care should be taken to close it tightly again.
- Opened kits retain activity for 4 weeks if stored as described above. The Precipitation Reagent has to be stored protected from light.
- If crystals are observed in the 40X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- Do not let wells dry during assay; add reagents immediately after completing the rinsing steps.
- Once the test has been started, all steps should be completed without interruption.
- Allow the reagents to reach room temperature (21°C - 26°C) before

starting the test. Temperature will affect the absorbance readings of the assay. However, values for the patient samples will not be affected.

- It is highly recommended that the standards, samples and controls be assayed in duplicates.
- Pipetting of samples and reagents must be done as quickly as possible and in the same sequence for each step.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Plasma: Whole blood should be collected into centrifuge tubes containing anti-coagulant (EDTA) and centrifuged immediately after collection. (E.g. for plasma Sarstedt Monovette with EDTA; 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 (≥ 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.

Stability of plasma sample:

15°C – 25°C for 2 hours

2°C – 8°C for 8 hours

- 20°C for 1 month

* Thawed samples should be inverted several times prior to testing.

Urine: In urine C5a is stable at room temperature (15°C to 25°C) for 24 hours. 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.

Stability of urine sample:

15°C- 25°C for 24 hours

2°C – 8°C for 3 days (When stored separately from pellet)

Note:

a) Haemolytic and lipaemic plasma and plasma containing rheumatoid factors do not interfere with the assay. However, we still recommended do not use haemolytic, icteric or lipaemic specimens.

b) Samples containing sodium azide should not be used in the assay.

REAGENT PREPARATION

- **Standard and Control**: Reconstitute the lyophilized contents of the standard vial with **1.0 mL deionized water**.

  Note: The reconstituted standards and control can be used within 8 hours at 15°C to 25°C or within 1 day at 2°C to 8°C. For longer storage aliquot and freeze at -20°C for up to 4 weeks. Frozen (-20°C) reconstituted Standards or Control should only be used once within 4 weeks. .
- **1X Wash buffer**: Add deionized water to the **40X** concentrated 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.
- **Enzyme Conjugate antibody:** Pipette **200 µL** of **HRP-conjugated Anti-human C5a antibody** into a vial of **Antibody Diluent (11 mL)** and shake gently to mix (sufficient for 1 test plate). Working Conjugate Solution can be stored at 2°C to 8°C for 4 weeks.
- **Sample Diluent:** Prepare a Dilution Reagent by mixing an equal volume of Tris buffer ((Tris/HCl Buffer pH 8.0 Tris (100 mmol/L), NaCl (25 mmol/L)) with distilled water.
- **Sample Dilution:** If in an initial assay, a specimen is found to contain more than the highest standard or if high values are expected dilute the plasma sample first 1:10 with **Sample Diluent** and then apply the normal precipitation step to the diluted sample. For the calculation of the concentrations this dilution factor has to be taken into account.

SAMPLE 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 according to the steps below:

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 µL of **sample** and 100 µL of **Precipitation Reagent** is recommended.
2. Mix intensively at once and incubate at least for **3 min**. at **15°C – 25°C**.
3. Centrifuge the mixture for **10 min**. at **~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°C – 25°C for 1 day and at 2°C – 8°C for 3 days if stored separately from the pellet.

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT) before use. Standards, samples and controls should be assayed in duplicates.

1. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.

   For Plasma Samples:

2. Add **50 µl** of **Assay Buffer** into each well.
3. Add **50 µl** of **precipitated standards, control or unknown samples** into appropriate wells. Shake plate briefly to ensure thorough mixing.
4. Incubate for **20 minutes at RT** (20 °C – 25 °C).
5. Aspirate each well and wash, repeating the process 2 times for a total **3**

washes. Wash by filling each well with **1× wash buffer (300 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining buffer by aspirating, decanting or blotting against clean paper towels. (The sensitivity and precision of this assay is markedly influenced by the correct performance of the washing procedure!)

6. Add **100 µl** of **working Enzyme conjugate antibody** into each well. Incubate for **15 minutes at RT** (20 °C – 25 °C).
7. **Wash** as according to **step 5**.
8. Add **100 µl** of **TMB Reagent** to each well. Incubate for **13-17 minutes at RT**. **(Protect from light)**
9. Add **100 µl** of **Stop Solution** to each well.
10. Read the OD with a microplate reader at **450 nm** immediately. It is recommended that the wells be read within 10 minutes after adding the Stop Solution.

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/L 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 "Assay Procedure for Plasma Samples".

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and patient samples.

2. Using linear graph paper, 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.

3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

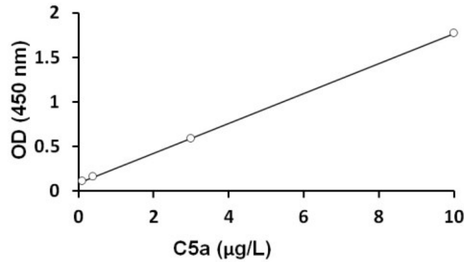
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

5. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for the detail. (https://www.arigobio.com/elisa-analysis)

6. 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 further diluted or reported as such. For the calculation of the concentrations this dilution factor has to be taken into account.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

Assay range: 0.02 – 10.0 µg/L

Minimum Detectable Concentration: < 0.02 µg/L

Expected Values

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

Preliminary Reference Interval:

The Normal Values 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

The results alone should not be the only reason for any therapeutic consequences. The results should be correlated to other clinical observations and diagnostic tests.

Specificity

This kit also cross-react to C5a from rabbit, pig and rhesus monkey samples.

Intra-assay and Inter-assay precision

The CV values of intra-assay was 5-8% and inter-assay was 6-10%.

Recovery

86 - 114% (plasma samples)