DRG® C5a ELISA (EIA-3327)

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1. INTRODUCTION

The DRG **C5a** Enzyme Immunoassay Kit provides materials for determination of Anaphylatoxin C5a in human plasma or urine. This kit is intended for Research Use Only.

2. PRINCIPLE OF THE TEST

The **DRG C5a ELISA KIT** is an enzyme immunoassay for 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 appox. 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

- 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_2SO_4. 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 directly from DRG International, Inc.
  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_2SO_4
   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 at DRG, product code: EIA-3327-BUF)
2. Centrifuge: suited for small reaction tubes (e.g. Eppendorf).
3. A microtiterplate calibrated reader (450±10 nm)(e.g. the DRG Instruments Microtiterplate Reader).
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, DRG have 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 (≥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 **plasma** 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 **urine** sample: +15 to + 25°C 24 hours

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 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 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 crossconamination
- 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

- 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 donor 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.

10. REFERENCES

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