Human C3a

DPG-C3A

ELISA KIT
INSTRUCTIONS FOR USE

For Research Use Only
Not for clinical or diagnostic use

Diapharma Group, Inc., 8948 Beckett Road, West Chester, OH 45069 USA www.diapharma.com info@diapharma.com
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1. INTENDED USE

The human C3a ELISA kit is for use the *in vitro* quantitative determination of human C3a/C3a-desArg in serum, plasma, bronchoalveolar lavage (BAL) fluid, or urine samples. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic protocols.

The analysis should be performed by trained laboratory professionals.

2. INTRODUCTION

The complement system is an important factor in innate immunity. It also interacts with and is affected by the activated coagulation system. Notably, complement is a highly regulated system controlled by both membrane bound and soluble inhibitors. Hyperactivation of complement due to loss of control mechanisms is often associated with thrombotic disease.

Complement component 3 (C3) is a key protein of the complement system. During its activation, C3 is proteolytically cleaved by C3 covertase and the activation peptide, C3a, a small polypeptide of 77 amino acids (10kDa), is generated.

C3a induces acute inflammation by activating mast cells and neutrophils. Its pro-inflammatory actions modulate both innate and adaptive immune responses.

C3a itself is short-lived and in serum it is rapidly cleaved into the more stable, though biologically active, C3a-desArg. Measurement of C3a-desArg, via a capture antibody that recognizes a neo-epitope, informs about the level of complement activation in test samples.

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3. KIT FEATURES

- 3½ hour processing time
- Measurable concentration range: 31.3 - 2000 pg/ml.
- Sample volume required: 100 µl/well

Cross-reactivity

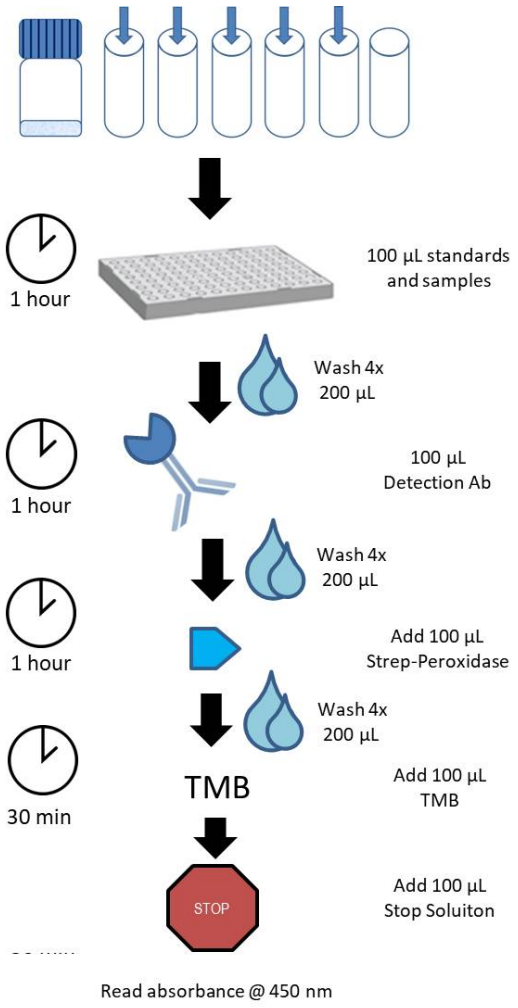
Potential cross-reacting proteins detected in the human C3a ELISA:

Cross reactant	Reactivity
Human C5a	**No**
Swine	**No**

Table 1

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

4. PROTOCOL OVERVIEW



- The human C3a ELISA is a ready-to-use, solid-phase enzyme-linked immunosorbent assay based on the sandwich principle.

- Microtiter wells are coated with antibodies that capture human C3a.
- Biotinylated tracer antibody binds to the captured human C3a.
- Streptavidin-peroxidase conjugate binds to the biotinylated tracer antibody.
- Streptavidin-peroxidase conjugate reacts 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 C3a standards (log).
- The human C3a concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.

5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Kit component	Quantity DPG-C3A	Color code
Wash buffer 20x	1 vials (60 ml)	**Colorless**
Dilution buffer 10x	1 vial (20 ml)	**Green**
Standard	2 vials, lyophilized	**White**
Tracer, biotinylated	1 vial, 1 ml lyophilized	**White**
Streptavidin-peroxidase 100x	1 tube, 0.25 ml in solution	**Brown**
TMB substrate	1 vial (11 ml)	**Brown**
Stop solution	1 vial (22 ml)	**Red**
12 Microtiter strips, pre-coated	1 plate	
Certificate of analysis	1	
Manual	**1**	

Table 2

- Upon receipt, store individual components at 2 - 8°C. Do not freeze.
- Do not use components beyond the expiration date printed on the label.
- When stored at 2 - 8°C, the standard and tracer in lyophilized form and the streptavidin-peroxidase in concentrated solution are stable until the expiration date indicated on the label.
- The exact amount of the standard is indicated on the label of the vial and in the Certificate of Analysis.
- Once reconstituted, the standard is ready to use for a single use and cannot be stored.
- Once reconstituted, the tracer is stable for 1 month if stored at 2 - 8°C.
- The streptavidin-peroxidase can only be stored in concentrated solution and is not stable when stored dilute.
    - Upon receipt, the foil pouch surrounding the plate should be vacuum-sealed and free from punctures. Any irregularities to aforementioned conditions may influence plate performance in the assay.
    - Return unused strips immediately to the foil pouch containing the desiccant pack and reseal along the entire edge of the zip-seal. Quality is guaranteed for 1 month if stored at 2 - 8°C.

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
- Centrifuge for 1 ml tubes

6. WARNINGS AND PRECAUTIONS

- For research use only, not for diagnostic or therapeutic use.
- This kit should only be used by qualified laboratory staff.
- Do not ingest any of the kit components.
- The standard is of human origin. It has been tested for various viruses and found negative. Since no test method can offer complete assurance that infectious agents are absent, this reagent should be handled as any potentially infectious human serum or blood specimen. Handle all materials in contact with this reagent according to guidelines for prevention of transmission of blood-borne infections.
- Handle all biological samples as potentially hazardous and capable of transmitting diseases.
- Kit reagents contain 2-chloroacetamide as a preservative. 2-Chloroacetamide is harmful to the skin and toxic if swallowed. In the case of an accident or if you feel unwell, seek medical attention immediately.
- The stop solution contains 2% oxalic acid and can cause irritation or burns to respiratory system, skin and eyes. Direct contact with skin and eyes should be strictly avoided. If contact occurs, rinse immediately with plenty of water and seek medical attention.
- Do not under any circumstances add sodium azide as a preservative to any of the components.
- Open vials carefully: vials are under vacuum.
- It is advised to spin down streptavidin-peroxidase tubes before use.
- The TMB substrate is light sensitive and should be kept away from bright light. The solution should be colorless until use.
- Do not use kit components beyond the expiration date.
- Do not mix reagents from different lots. The reagents have been standardized as a unit for a given lot. Use only the reagents supplied by the manufacturer.
- Do not reuse microwells or pour reagents back into their bottles once dispensed.
- Use polypropylene tubes for preparation of standard and samples. Do not use polystyrene tubes or sample plates.
- The assay has been optimized for the indicated standard range. Do not change the standard range.
- Incubation times, incubation temperature and pipetting volumes other than those specified may give erroneous results.
- Hemolyzed, hyperlipemic, heat-treated or contaminated samples may give erroneous results.

7. SAMPLE PREPARATION

Collection and handling

Serum or plasma

It is critical that sample collection is performed correctly. Take care to avoid C3a generation in the samples. All specimen handling operations should be carried out at 4°C for plasma and serum (immediately after clotting). The most reliable results will be obtained if heparin or EDTA plasma is used.

Serum

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

Plasma

Keep freshly collected blood on ice. Within 20 minutes after blood sampling, separate plasma by centrifugation (1500xg at 4°C for 15 min). Transfer the plasma to a fresh polypropylene tube.

Bronchoalveolar lavage (BAL) fluid

Perform BAL during bronchoscopy by standardized washing of the segment. Aspirate each aliquot of physiologic fluid immediately after inspiration. Discard the first aliquot of recovered BAL fluid. Collect the BAL fluid in polypropylene tubes and keep on ice. Separate cells from BAL fluid by centrifugation (500xg at 4°C for 5 min). Filter cell-free BAL fluid through a layer of gauze to remove mucus strands.

Urine

Collect urine using normal aseptic technique. Centrifuge the urine to remove debris (1500xg at 4 °C for 15 min). Transfer urine to a fresh polypropylene tube.

Storage

Store samples at -70°C in polypropylene tubes. Avoid multiple freeze-thaw cycles which may influence the reliability of human C3a measurement and give erroneous results.

Use samples within 6 hours after thawing.

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 C3a can be measured accurately if serum samples are diluted at least 4000x with supplied dilution buffer in polypropylene tubes.

Plasma samples

Human C3a can be measured accurately if plasma samples are diluted at least 300x with supplied dilution buffer in polypropylene tubes.

BAL fluid samples

Human C3a can be measured accurately if BAL fluid samples are diluted at least 5x with supplied dilution buffer in polypropylene tubes.

Urine samples

Human C3a can be measured accurately if urine samples are diluted at least 4x with supplied dilution buffer in polypropylene tubes.

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 C3a from an undilute sample is not 100% and may vary from sample to sample. When testing less dilute samples, it is advisable to run recovery experiments to determine the influence of the matrix on the detection of human C3a.

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

Guideline for dilution of samples

See the table below for recommended sample dilutions. Volumes are based on a total volume of at least 230 µl of dilute sample, which is sufficient for one sample in duplicate in the ELISA. For dilution of samples we recommend using 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 3

8. REAGENT PREPARATION

Allow all the reagents to equilibrate to room temperature (20 – 25°C) prior to use. Return reagents 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 20 ml of the 10x dilution buffer with 180 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.

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 1. Prepare each human C3a

standard in polypropylene tubes by serial dilution of the reconstituted standard with dilution buffer as shown in Figure 1*. After reconstitution the standard cannot be stored for repeated use.

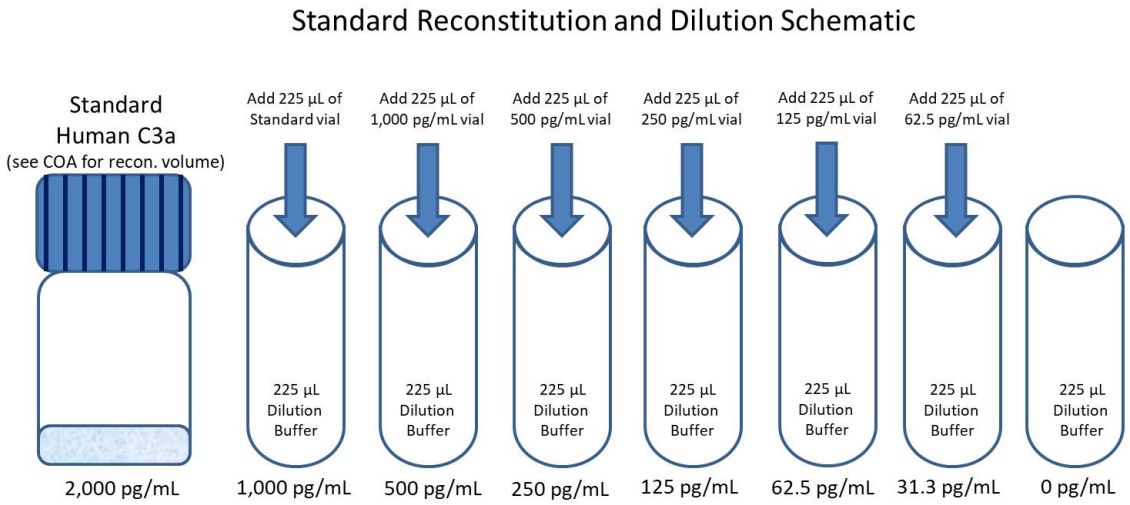


Figure 1

Tracer solution

The tracer is reconstituted by pipetting 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 advisable 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.

9. ELISA PROTOCOL

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

1. Determine the number of test wells required, place 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.
2. Transfer 100µl of standard, samples, or controls into appropriate wells. Pipette all standards, samples and controls in duplicate. 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 plate for 1 hour at room temperature.
5. Wash the plates 4 times with wash buffer as follows*:
   a. Carefully 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 1x 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 a 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 monitor 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 the solution 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.

*) If a 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.

10. INTERPRETATION OF RESULTS

- Calculate the mean absorbance for each set of duplicate standards, control and samples.
- If individual absorbance values differ by more than 15% from the corresponding mean value, the result is considered suspect and the sample should be retested.
- The mean absorbance of the zero standard should be less than 0.3.
- Create a standard curve using computer software capable of generating a good curve fit. The mean absorbance for each standard concentration is plotted on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis (logarithmic scale).
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
- Samples that give a mean absorbance above the absorbance for the highest standard concentration are out of range of the assay. These samples should be retested at a higher dilution.

11. TECHNICAL HINTS

- User should be trained and familiar with ELISA assays and test procedure.
- If you are not familiar with the ELISA technique it is recommended to perform a pilot assay prior to evaluation of samples. Perform the assay with a standard curve only following the instructions.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing wash buffer, fill with wash buffer as indicated for each cycle and do not allow wells to sit uncovered or dry for extended periods.
- Since exact conditions may vary from assay to assay, a standard curve must be established for each run.
- Do not mix reagents from different batches, or other reagents and strips. Remainders should not be mixed with contents of freshly opened vials.
- Each time the kit is used, fresh dilutions of standard, sample, tracer, streptavidin-peroxidase and buffers should be made.
- Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
- To avoid cross-contaminations, change pipette tips between reagent additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- The waste disposal should be performed according to your laboratory regulations.

12. QUALITY CONTROL

The Certificate of Analysis included in this kit is lot specific and is to be used to verify results obtained by your laboratory. The absorption values provided on the Certificate of Analysis are to be used as a guideline only. The results obtained by your laboratory may differ.

This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the immunoassay, the possibility of interference cannot be excluded.

For optimal performance of this kit, it is advisable to work according to good laboratory practice.

13. TROUBLESHOOTING

Warranty claims and complaints in respect of deficiencies must be logged before the expiry date of the product.

Contact **DPGtechsupport@diapharma.com** with questions or help troubleshooting.

14. REFERENCES

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