ab136937 – Complement C4a des Arg Human ELISA Kit

Instructions for Use

For quantitative detection of Human Complement C4a des Arg in Plasma.

This product is for research use only and is not intended for diagnostic use.

Version 4 Last Updated 5 February 2019

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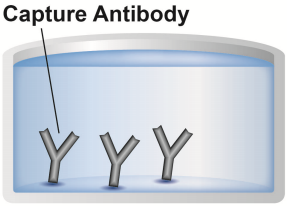
1. BACKGROUND

Abcam's Human Complement C4a des Arg *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of Complement C4a des Arg in Human plasma samples.

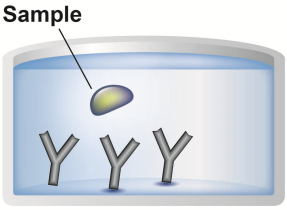
A goat anti-rabbit IgG antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with an alkaline phosphatase (AP) conjugated-Complement Complement C4a des Arg antigen and a polyclonal antibody specific to Complement C4a des Arg. After incubation the excess reagents are washed away and pNpp substrate is added, which causes generation of yellow color. The intensity of the yellow coloration is inversely proportional to the amount of Complement C4a des Arg captured in the plate.

The Human Complement C4a des Arg molecule is one of three activation fragments formed from the activation of the complement cascade. Complement C4a des Arg is formed from C4a via carboxypeptidase cleavage of the C-terminal arginine group. Human Complement C4a des Arg contains 76 amino acids with 6 cysteines involved in disulfide bridges. The structure of Complement C4a des Arg in Human, cow, rat, and mouse is similar. Complement C4a des Arg is a highly cationic molecule containing no carbohydrate. C4a is a potent constrictor of smooth muscle cells, and has been shown to increase vascular permeability. The long term study of liver and other transplant recipients for both C3a des Arg and Complement C4a des Arg may be useful in assessing a number of pathological conditions. The use of potent protease inhibitors, such as Futhan, in conjunction with EDTA, may allow complement activation factors to be quantitated specifically via inhibition of non-specific protease formation of Complement C4a des Arg.

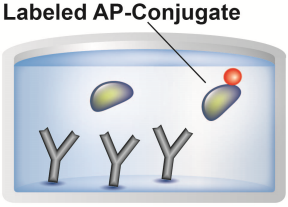
2. ASSAY SUMMARY



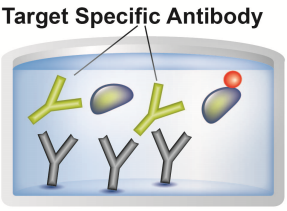
Prepare all reagents and samples as instructed.



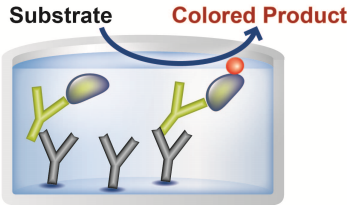
Add standards and samples to appropriate wells.



Add prepared labeled AP-conjugate to appropriate wells.



Add Complement C4a des Arg antibody to appropriate wells. Incubate at room temperature.



Add pNpp substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up
- Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use
- The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA
- We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results
- The Human Complement C4a des Arg Standard provided is supplied in ethanolic buffer at a pH optimized to maintain human Complement C4a des Arg integrity. This material is derived from Human serum tested negative for HIV and Hepatitis, but should be treated as potentially infectious

4. STORAGE AND STABILITY

Store kit at +4°C immediately upon receipt, apart from the AP Conjugate & Standard, which should be stored at -20°C. Avoid multiple freeze-thaw cycles.

Refer to list of materials supplied for storage conditions of individual components.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Goat anti-rabbit IgG Microplate (12 x 8 wells)	96 Wells	+4°C
Human Complement C4a des Arg Alkaline Phosphatase Conjugate	6 mL	-20°C
Human Complement C4a des Arg Antibody	6 mL	+4°C
Human Complement C4a des Arg Standard	500 µL	-20°C
Assay Buffer 10 Concentrate	15 mL	+4°C
20X Wash Buffer Concentrate	30 mL	+4°C
pNpp Substrate	20 mL	+4°C
Stop Solution	6 mL	+4°C
Complement Reagent A	15 mL	+4°C
Complement Reagent B	30 mL	+4°C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Standard microplate reader - capable of reading at 405 nm, preferably with correction between 570 and 590 nm.
- Automated plate washer (optional)
- Adjustable pipettes and pipette tips. Multichannel pipettes are recommended when large sample sets are being analyzed
- Eppendorf tubes
- Microplate Shaker
- Absorbent paper for blotting
- Deionized water
- 9.0 N NaOH and 10.0 HCl for plasma precipitation

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Standards can be made up in either glass or plastic tubes
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent
- Pipette standards and samples to the bottom of the wells
- Add the reagents to the side of the well to avoid contamination
- This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided
- Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands
- Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18 - 25°C) prior to use.

9.1 Human C4a Alkaline Phosphatase Conjugate

Allow the C4a Alkaline Phosphatase Conjugate to equilibrate to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20°C.

9.2 1X Wash Buffer

Prepare the 1X Wash Buffer by diluting 5 mL of the 20X Wash Buffer Concentrate in 95 mL of deionized water. Mix thoroughly and gently.

9.3 1X Assay Buffer 10

Prepare Assay Buffer 10, 1X by diluting 10 mL of the supplied concentrate with 90 mL deionized water. This can be stored at room temperature until the expiration date, or for 3 months, whichever is earlier.

9.4 Conjugate 1:10 Dilution for Total Activity Measurement

Prepare the Conjugate 1:10 Dilution by diluting 50 µL of the supplied conjugate with 450 µL of 1X Assay Buffer 10. This dilution should be used within 3 hours of preparation. This 1:10 dilution is intended for use in Total Activity wells only.

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use. Diluted standards should be used within 60 minutes of preparation.

10.1 Allow the 2,000 ng/mL Human Complement C4a des Arg **Stock Standard** solution to equilibrate to room temperature. The standard solution should be stored at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles

10.2 Label five tubes with numbers #1 – #5.

10.3 Add 900 µL of appropriate diluent (Assay Buffer or Tissue Culture Media) to tube #1.

10.4 Add 750 µL appropriate diluent to tubes #2 through #5

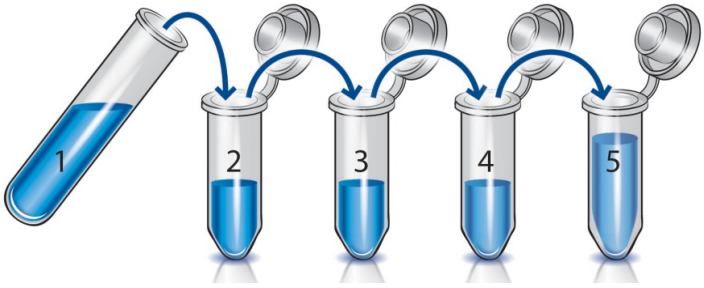
10.5 Prepare a 200 ng/mL **Standard 1** by adding 100 µL of the 2,000 ng/mL Stock Standard to tube #1. Vortex thoroughly.

10.6 Prepare **Standard 2** by transferring 250 µL from Standard 1 to tube 2. Vortex thoroughly.

10.7 Prepare **Standard 3** by transferring 250 µL from Standard 2 to tube 3. Vortex thoroughly.

10.8 Using the table below as a guide, repeat for tubes #4 and #5.

Standard #	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Standard	100	900	2,000	200
2	Standard 1	250	750	200	50
3	Standard 2	250	750	50	12.5
4	Standard 3	250	750	12.5	3.13
5	Standard 4	250	750	3.13	0.78



11. SAMPLE COLLECTION AND STORAGE

- The Complement C4a des Arg Human kit is compatible with Complement C4a des Arg samples in EDTA/Futhan tube which have undergone the following procedure.

11.1 Sample Collection

To collect blood, use EDTA/Futhan tubes (if not available, use EDTA tubes). Collect blood in a 7 mL tube and centrifuge for 15 minutes at 2,000 x g at 4°C. Assay plasma immediately or store on ice for up to six hours. Aliquots (225 µL) of plasma collected in EDTA/Futhan tubes may be stored at ≤ - 70°C.

Note: Collect blood in EDTA/Futhan to avoid possible low-level complement activation.

11.2 Precipitating Plasma

11.2.1 Aliquot 225 µL volumes of plasma into 1.5 to 2 mL microcentrifuge tubes. Use immediately or store at ≤-70 °C for long term storage.

11.2.2 Add 225 µL of Complement Reagent A to each sample and vortex thoroughly.

11.2.3 Add 50 µL of 10.0 N HCl to each sample, vortex thoroughly, and incubate at room temperature for 1 hour

11.2.4 During the 1 hour incubation, prepare Assay Buffer 10.

11.2.5 Spin the samples at 10,000 rpm in a microcentrifuge at room temperature for 5 minutes. Transfer 180 µL of the supernatant into a clean, plastic test tube.

11.2.6 To this supernatant, add 20 µL of 9.0 N NaOH and vortex thoroughly.

11.2.7 Add 600 µL of Complement Reagent B to the supernatant and vortex thoroughly.

11.2.8 Add 10.7 µL of Assay Buffer 10 to the supernatant and vortex thoroughly (This addition will ensure that the sample has been diluted 1:10 fold).

11.2.9 Dilute all samples 1:20 fold in Assay Buffer 10 prior to running the assay. In fresh plastic tubes, dilute 50 µL of each sample with 950 µL of Assay Buffer 10. Mix each tube thoroughly.

- Human samples diluted using this recommended procedure will read within the standard curve. Some samples may read too high and may require a further 1:2 to 1:10 dilution to be accurately determined.

- When quantifying complement levels, be sure to correct sample values to take into account dilution factors from all steps. There will be a 1:200 dilution of all plasma samples when the steps above are followed

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at 4°C
- For each assay performed, a minimum of 2 wells must be used as blanks, omitting primary antibody from well additions
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay.

	1	2	3	4
A	B_s	Std 1	Std 5	
B	B_s	Std 1	Std 5	
C	TA	Std 2	Sample 1	
D	TA	Std 2	Sample 1	
E	NSB	Std 3	Sample 2	
F	NSB	Std 3	Sample 2	
G	B_0	Std 4	etc	
H	B_0	Std 4	etc	

Plate layout shows controls, blanks and standards required for each assay. Use additional strips of wells to assay all your samples.

Key:

B_s = Blank; contains substrate only.

TA = Total Activity; contains conjugate (5 µL) and substrate.

NSB = Non-specific binding; contains standard diluent, assay buffer, conjugate and substrate.

B_0 = 0 pg/mL standard; contains standard diluent, conjugate, antibody and substrate

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use**
- **It is recommended to assay all standards, controls and samples in duplicate**
- **Refer to the recommended plate layout in Section 12 before proceeding with the assay**

13.1 Add 150 µL appropriate diluent* into the NSB (non-specific binding) wells. (*Use the same diluent used to prepare standards in section 10, either Assay Buffer or Tissue Culture Media).

13.2 Add 100 µL appropriate diluent (Assay Buffer or tissue culture media) into the B_0 (0 pg/mL standard) wells.

13.3 Add 100 µL of standards and 100 µL diluted samples into the appropriate wells.

13.4 Invert the bottle of Complement C4a des Arg Alkaline Phosphatase Conjugate (blue) 4-5 times and add 50 µL into each well, except the Total Activity (TA) and the B_s wells.

13.5 Add 50 µL of Complement C4a des Arg antibody (yellow) into B_0, standard and sample wells, i.e. not B_s, TA and NSB wells.

Note: Every well used should be green in color except the NSB wells which should be blue. The B_s and TA wells are empty at this point and have no color.

13.6 Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided.

13.7 Empty the contents of the wells and wash by adding 400 µL of 1X Wash Buffer to every well. Repeat the wash 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

13.8 Add 5 µL of the 1:10 Alkaline Phosphatase Conjugate to the TA wells. Agitating gently before use.

13.9 Add 200 µL of the pNpp Substrate solution to every well. Incubate at 37°C for 1 hour without shaking.

13.10 Add 50 µL Stop Solution into each well. The plate should be read immediately.

13.11 Read the O.D. absorbance at 405 nm, preferably with correction between 570 and 590 nm.

14. CALCULATIONS

14.1 Calculate the average net absorbance measurement (Average Net OD) for each standard and sample by subtracting the average NSB absorbance measurement from the average absorbance measurement (Average OD) for each standard and sample.

Average Net OD = Average Bound OD - Average NSB OD

14.2 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula

$$\text{Percent Bound} = \frac{\text{Average Net OD}}{\text{Average Net } B_0 \text{ OD}} \times 100$$

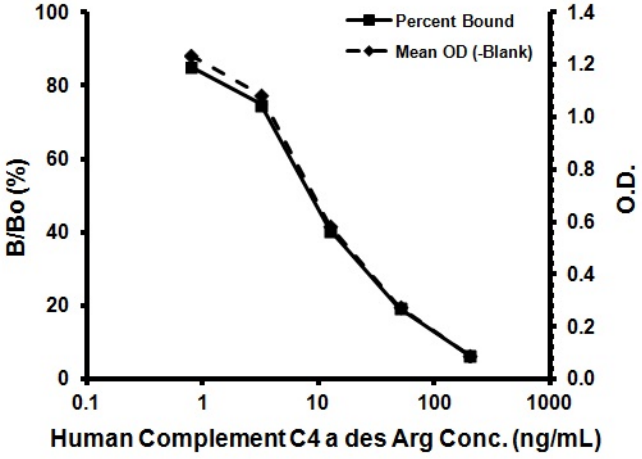
14.3 Plot the Percent Bound (B/B_0) and the net OD versus concentration of Complement C4a des Arg for the standards. The concentration of Complement C4a des Arg in the unknowns can be determined by interpolation of net OD values.

A four parameter algorithm (4PL) provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted.

Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Sample	Mean OD ($-B_s$)	% Bound	Complement C4a des Arg ng/mL
B_s OD	(0.122)	-	-
TA	0.892	-	-
NSB	-0.003	-	-
Standard 1	0.091	6.5	200
Standard 2	0.279	19.4	50
Standard 3	0.587	40.6	12.5
Standard 4	1.086	74.9	3.13
Standard 5	1.235	85.2	0.78
B_0	1.424	100	0
Unknown1	0.187	13.1	73
Unknown 2	0.620	42.9	12.4

Typical Quality Control Parameters

Total Activity Added	= 1.0 x 10 x 10 = 101.4
%NSB	= 0.1%
$\%B_0/TA$	= 1.5%
Quality of Fit	= 1.0000 (Calculated from 4 parameter logistic curve fit)
20% Intercept	= 40.0 ng/mL
50% Intercept	= 9.65 ng/mL
80% Intercept	= 2.10 ng/mL

16. TYPICAL SAMPLE VALUES

SENSITIVITY –

The sensitivity, minimum detectable dose of Complement C4a des Arg using this Abcam ELISA kit was found to be 0.76 ng/mL. This was determined by the average optical density of the 0 pg/mL Standard and comparing to the average optical density for Standard #5. The detection limit was determined as the concentration of Complement C4a des Arg measured at two standard deviations from the zero along the standard curve.

LINEARITY OF DILUTION –

A sample containing 19.39 ng/mL Human Complement C4a des Arg was diluted 4 times 1:2 in the kit Assay Buffer 10 and measured in the assay. The data was plotted graphically as actual Human Complement C4a des Arg concentration versus measured Human Complement C4a des Arg concentration.

The line obtained had a slope of 0.973 and a correlation coefficient of 0.994

PRECISION –

Intra-Assay

	Human Complement C4a des Arg (ng/mL)	%CV
Low	2.2	13.9
Medium	5.0	10.5
High	10.8	6.7

Inter-Assay

	Human Complement C4a des Arg (ng/mL)	%CV
Low	5.13	12.5
High	10.1	11.3

17. ASSAY SPECIFICITY

CROSS REACTIVITY –

The cross reactivities for a number of related molecules was determined by dissolving the cross reactant (purity checked by analytical methods) in Assay Buffer 10 at concentrations from 100,000 to 0.1 ng/mL. These samples were then measured in the human C4a des Arg assay and the measured human C4a des Arg concentration at 50% B/B_0 calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage:

Compound	Cross Reactivity (%)
Human Complement C4a des Arg	100
Human Complement C4	2.14
Human Complement C5 des Arg	0.28
Human Complement C3	0.23
Human Complement C3a des Arg	0.04
Human Complement C5	0.02

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed

19. NOTES

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