

PRODUCT INFORMATION



Complement C3a des Arg (Human), EIA kit

Catalog No. IB09632

96 Well Kit

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Description

The Complement C3a des Arg (human), EIA kit is a competitive immunoassay for the determination of C3a des Arg in plasma. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to human C3a des Arg to bind, in a competitive manner, the human C3a des Arg in the sample or an alkaline phosphatase molecule which has human C3a des Arg attached to it. After incubation at room temperature the excess reagents are washed away and substrate is added. After a short incubation, the enzyme reaction is stopped and the yellow color generated is read on a microplate reader at 405nm. The intensity of the bound yellow color is inversely proportional to the concentration of human C3a des Arg in either standards or samples. The measured optical density is used to calculate the concentration of human C3a des Arg. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard¹ or Tijssen².

Introduction

The human C3a des Arg molecule is one of three activation fragments formed from the activation of the complement cascade. C3a des Arg is formed from C3a via carboxypeptidase cleavage of the C-terminal arginine group³⁻⁵. The structure of human C3a des Arg was first reported in 1975 by Hugli⁶. Human C3a des Arg contains 77 amino acids with 6 cysteines involved in disulfide bridges between residues 22-49, 23-56 and 36-57. The C-terminal end of C3a des Arg in human, porcine, rat, mouse and guinea pig is identical. C3a des Arg is a highly cationic molecule containing no carbohydrate. X-ray crystal data shows the N- and C-terminal 6-8 residues to have highly flexible helical structures⁷. C3a is one of the most potent constrictors of smooth muscle cells, and guinea pig airways are hyperresponsive to C3a when pretreated with histamine⁸. The long term study of liver and other transplant recipients for both C3a des Arg and C4a des Arg may be useful in assessing a number of pathological conditions⁹. The use of potent protease inhibitors, such as Futhan, in conjunction with EDTA, may allow complement activation factors to be determined specifically via inhibition of non-specific protease formation of C3a des Arg.

Precautions

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1. 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.
2. Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg²⁺ and Zn²⁺ ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
4. 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.
5. The human C3a des Arg Standard provided, Catalog No. 80-0633, is lyophilized at a pH optimized to maintain C3a des Arg integrity. This material is derived from human serum tested negative for HIV and Hepatitis, but should be treated as potentially infectious.

Materials Supplied

- 1. Goat anti-Rabbit IgG Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0060**
A plate using break-apart strips coated with goat antibody specific to rabbit IgG.
- 2. human C3a des Arg EIA Conjugate, 6 mL, Catalog No. 80-0632**
A blue solution of alkaline phosphatase conjugated with C3a des Arg.
- 3. human C3a des Arg EIA Antibody, 6 mL, Catalog No. 80-0634**
A yellow solution of a rabbit polyclonal antibody to C3a des Arg.
- 4. Assay Buffer 10 Concentrate, 15 mL, Catalog No. 80-0668**
Tris buffer with additives and preservatives
- 5. Wash Buffer Concentrate, 30 mL, Catalog No. 80-1286**
Tris buffered saline containing detergents.
- 6. human C3a des Arg Standard, 500 ng, Catalog No. 80-0633**
2 vials containing 500 ng each of lyophilized C3a des Arg.
- 7. Complement Reagent A, 15 mL, Catalog No. 80-0637**
- 8. Complement Reagent B, 30 mL, Catalog No. 80-0638**
- 9. pNpp Substrate, 23 mL, Catalog No. 80-0075**
A solution of p-nitrophenylphosphate in buffer. Ready to use.
- 10. Stop Solution, 6 mL, Catalog No. 80-0247**
A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
- 11. C3a des Arg Assay Layout Sheet, 1 each, Catalog No. 30-0122**
- 12. Plate Sealers, 2 each, Catalog No. 30-0012**

Storage

All components of this kit, **except the conjugate and lyophilized standard**, are stable at 4°C until the kit's expiration date. The human C3a des Arg Conjugate and lyophilized Standard should be stored at -20°C upon receipt.

Materials Needed but Not Supplied

1. Deionized or distilled water. No difference in assay results is seen with distilled water.
2. Precision pipets for volumes between 5 μ L and 1,000 μ L.
3. Repeater pipets for dispensing 50 μ L and 200 μ L.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.
9. 9.0 N NaOH and 10.0 N HCl for plasma precipitation.

Sample Handling

The Complement C3ades Arg (human) EIA kit is compatible with human C3ades Arg plasma samples in EDTA/Futhan tubes which have undergone the following procedure. **All plasma samples must be treated prior to running the assay using the procedure below.**

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 may be stored at ≤ -70 °C.

NOTE: Collect blood in EDTA/Futhan to avoid possible low-level complement activation. Handle and dispose of all specimens as if they are capable of transmitting infectious agents.

2. Precipitating Plasma

The following protocol precipitates whole protein from plasma. Whole protein in the sample competes with the complement in the assay.

- 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.
- 2.2 Add 225 µL of Complement Reagent A to each sample and vortex thoroughly.
- 2.3 Add 50 µL of 10.0 N HCl to each sample, vortex thoroughly, and incubate at room temperature for 1 hour.
- 2.4 During the 1 hour incubation, prepare Assay Buffer 10 according to directions on Page 5.
- 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.
- 2.6 To this supernatant, add 20 µL of 9.0 N NaOH and vortex thoroughly.
- 2.7 Add 600 µL of Complement Reagent B to the supernatant and vortex thoroughly.
- 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).
- 2.9 Dilute all samples 1:20 fold in Assay Buffer 10 prior to running the assay. In fresh tubes, dilute 50 µL of each sample with 950 µL of Assay Buffer 10. Vortex 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 determining 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.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. 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.
8. **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.
9. **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.**

Reagent Preparation

1. Assay Buffer 10, 1x

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

2. human C3a des Arg Standard

Allow the 500 ng human C3a des Arg Standard to warm to room temperature. Reconstitute 1 vial of 500 ng human C3a des Arg Standard with 500 μ L Assay Buffer 10. Vortex gently. Label seven 12x75 mm tubes #1 through #7. Pipet 1 mL of Assay Buffer 10 into tube #1. Pipet 500 μ L of Assay Buffer 10 into tubes #2 - #7. Remove 20 μ L of Assay Buffer 10 from tube #1. Add 20 μ L of the 1000 ng/mL stock solution to tube #1. Vortex thoroughly. Add 500 μ L of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #7.

The concentration of C3a des Arg in tubes #1 through #7 will be 20, 10, 5, 2.5, 1.25, 0.625 and 0.313 ng/mL respectively. See C3a des Arg Assay Layout Sheet for dilution details. Discard any remaining reconstituted human C3a des Arg standard.

Diluted standards should be used within 60 minutes of preparation.

3. human C3a des Arg Conjugate

Allow the conjugate to warm to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20°C. Avoid repeated freeze-thaws of the aliquots.

4. Conjugate 1:10 Dilution for Total Activity Measurement

Prepare the Conjugate 1:10 Dilution by diluting 50 μ L of the supplied conjugate with 450 μ L of Assay Buffer 10, 1x. This dilution should be used within 3 hours of preparation. This 1:10 dilution is intended for use in the Total Activitywells only.

5. Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.
2. Pipet 100 µL of Assay Buffer 10 into the NSB and the Bo (0 ng/mL Standard) wells.
3. Pipet 100 µL of Standards #1 through #7 into the appropriate wells.
4. Pipet 100 µL of the 1:200 diluted Samples (**See Procedure on page 4**) into the appropriate wells.
5. Pipet 50 µL of Assay Buffer 10 into the NSB wells.
6. Invert bottle of blue Conjugate 4-5 times and pipet 50 µL into each well, except the Total Activity (TA) and Blank wells.
7. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.

NOTE: Every well used should be **Green** in color except the NSB wells which should be **Blue**. The Blank and TA wells are empty at this point and have no color.

8. 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, if so desired.
9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of **3 Washes**.
10. 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.
11. Add 5 µL of the 1:10 dilution of Conjugate (see Step 4 , Reagent Preparation, on page 5) to the TA wells, agitating gently before use.
12. Add 200 µL of the p-Npp Substrate solution to every well. Cover with the second plate sealer provided and incubate at 37 °C for 1 hour without shaking.
13. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
14. Blank the plate reader against the Blank wells, read the optical density at 405 nm., preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of C3a des Arg in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of human C3a des Arg can be calculated as follows:

1. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

$$\text{Average Net OD} = \text{Average Bound OD} - \text{Average NSB OD}$$

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

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

3. Using Logit-Log paper plot Percent Bound versus Concentration of human C3a des Arg for the standards. Approximate a straight line through the points. The concentration of human C3a des Arg in the unknowns can be determined by interpolation.

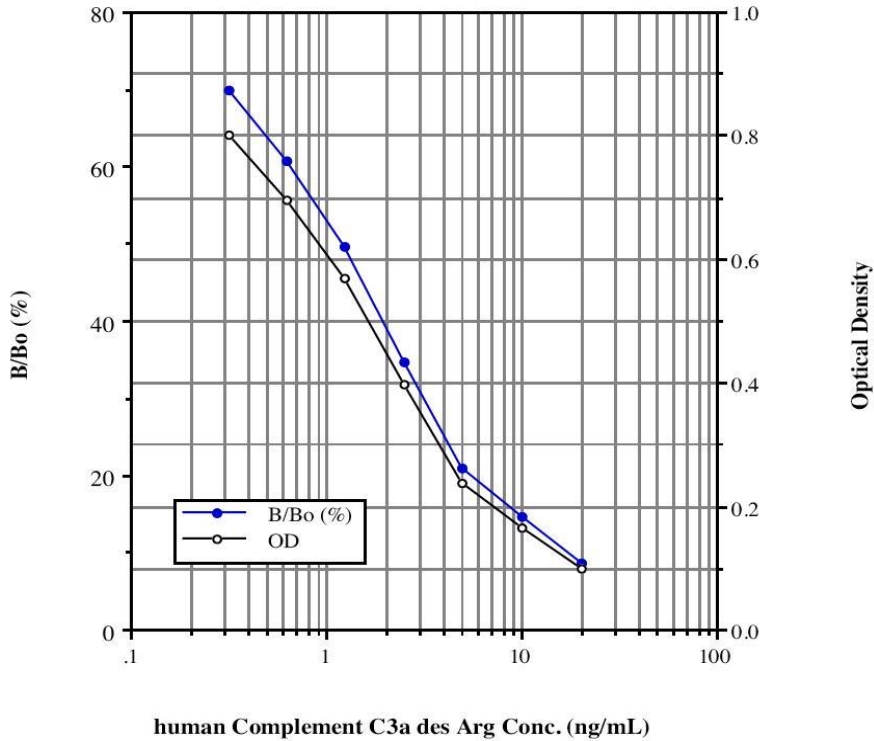
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

<u>Sample</u>	<u>Average OD</u>	<u>Net OD</u>	<u>Percent Bound</u>	<u>human C3a des Arg (ng/mL)</u>
Blank OD	(0.096)			
TA	0.233			
NSB	0.002	0		
Bo	1.145	1.143	100%	0
S1	0.098	0.096	8.6%	20.0
S2	0.167	0.165	14.6%	10.0
S3	0.239	0.237	20.9%	5.0
S4	0.399	0.397	34.8%	2.5
S5	0.568	0.566	49.6%	1.25
S6	0.695	0.693	60.7%	0.625
S7	0.802	0.800	70.0%	0.313
Unknown 1	0.345	0.343	30.0%	3.25
Unknown 2	0.555	0.553	48.4%	1.20

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate human C3a des Arg concentrations; each user must run a standard curve for each plate used.



Typical Quality Control Parameters

Total Activity Added	=	0.233 x 100 = 23.3
%NSB	=	0.175
%Bo/TA	=	4.91
Quality of Fit	=	0.9998 (Calculated from 4 parameter logistic curve fit)
20% Intercept	=	7.4 ng/mL
50% Intercept	=	1.2 ng/mL
80% Intercept	=	N/A

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols¹⁰.

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard #7. The detection limit was determined as the concentration of human C3a des Arg measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo	=	0.642
Average Optical Density for Standard #7	=	0.341
Delta Optical Density (0-0.313 ng/mL)	=	0.301
2 SD's of the Zero Standard	=	0.115
Sensitivity = $\frac{0.115}{0.301} \times 0.313 \text{ ng/mL}$	=	0.120 ng/mL

Linearity

A sample containing 4.86 ng/mL human C3a des Arg was diluted 3 times 1:2 in the kit Assay Buffer 10 and measured in the assay. The data was plotted graphically as actual human C3a des Arg concentration versus measured human C3a des Arg concentration.

The line obtained had a slope of 0.919 and a correlation coefficient of 0.989.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of human C3a des Arg and running these samples multiple times (n=16) in the same assay. Inter-assay precision is in process.

The precision numbers listed below represent the percent coefficient of variation for the concentrations of human C3a des Arg determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	<u>human C3a des Arg Concentration</u> (ng/mL)	<u>Intra Assay</u> %CV	<u>Inter Assay</u> %CV
Low	1.86	8.7	
Medium	3.40	9.8	
High	12.33	11.1	
Low	0.753		5.7
Medium	1.529		16.9
High	3.491		28.6

Cross Reactivities

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 C3a des Arg assay and the measured C3a des Arg concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

<u>Compound</u>	<u>Cross Reactivity</u>
human Complement C3a des Arg	100%
human Complement C3	1.28%
human Complement C4a des Arg	0.40%
human Complement C4	0.31%
human Complement C5 des Arg	0.10%
human Complement C5	0.05%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard Preparation.

Human Complement C3a des Arg concentrations were measured in human EDTA plasma. Human EDTA plasma was treated following the Sample Handling recommendation then spiked with human Complement C3a des Arg and assayed in the kit. The following results were obtained:

<u>Sample</u>	<u>% Recovery*</u>	<u>Recommended Dilution*</u>
human EDTA Plasma	94.8	-----

* See Sample Handling instructions on page 4 for details.

References

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