PRODUCT INFORMATION

human Complement C4a des Arg Enzyme Immunoassay Kit

Catalog No. IB09608

96 Well Kit

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Description

The IBL-America human Complement C4a des Arg kit is a competitive immunoassay for the determination of human C4a des Arg in plasma. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to human C4a des Arg to bind, in a competitive manner, the human C4a des Arg in the standard or sample or an alkaline phosphatase molecule which has human C4a 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 C4a des Arg in either standards or samples. The measured optical density is used to calculate the concentration of human C4a des Arg. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

The human C4a des Arg (C4a des Arg) molecule is one of three activation fragments formed from the activation of the complement cascade. C4a des Arg is formed from C4a via carboxypeptidase cleavage of the C-terminal arginine group[3-5]. The structure of human C4a des Arg was first reported in 1981 by Hugli[6]. Human C4a des Arg contains 76 amino acids with 6 cysteines involved in disulfide bridges. The structure of C4a des Arg in human, cow, rat, and mouse is similar. 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 permeablility[7]. 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 conditions[8]. The use of potent protease inhibitors, such as Futhan, in conjunction with EDTA, may allow complement activation factors to be detected specifically via inhibition of non-specific protease formation of C4a 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 Mg^{2+} and Zn^{2+} 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 C4a des Arg Standard provided is supplied in ethanolic buffer at a pH optimized to maintain human C4a 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**
   A plate using break-apart strips coated with goat antibody specific to rabbit IgG.
2. **human C4a des Arg EIA Conjugate, 6 mL**
   A blue solution of alkaline phosphatase conjugated with human C4a des Arg.
3. **human C4a des Arg EIA Antibody, 6 mL**
   A yellow solution of a rabbit polyclonal antibody to human C4a des Arg.
4. **Assay Buffer 10 Concentrate, 15 mL**
   Tris buffer with additives and preservatives.
5. **Wash Buffer Concentrate, 30 mL**
   Tris buffered saline containing detergents and a preservative.
6. **human Complement C4a des Arg Standard, 0.5 mL**
   A solution of 2,000 ng/mL human C4a des Arg.
7. **Complement Reagent A, 15 mL**
8. **Complement Reagent B, 30 mL**
9. **pNpp Substrate, 20 mL**
   A solution of p-nitrophenylphosphate in buffer. Ready to use.
10. **Stop Solution, 6 mL**
    A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
11. **human C4a des Arg Assay Layout Sheet, 1 each**
12. **Plate Sealers, 2 each**

Storage

All components of this kit, **except the conjugate**, are stable at 4°C until the kit's expiration date. The conjugate **must** be stored frozen at -20°C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. A 37 °C Incubator
9. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.
10. 9.0 N NaOH and 10.0 N HCl for plasma precipitation.

Sample Handling

This assay is compatible with human C4a des 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 collected in EDTA/Futhan tubes 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 supernatent 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 plastic 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 detecting 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 should be made up in 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 C4a des Arg Standard

Allow the 2,000 ng/mL human C4a des Arg Standard to warm to room temperature. Label five 12x75 mm tubes #1 through #5. Pipet 1 mL of Assay Buffer 10 into tube #1. Pipet 750 µL of Assay Buffer 10 into tubes #2 - #5. Remove 100 µL of Assay Buffer 10 from tube #1. Add 100 µL of the 2,000 ng/mL standard to tube #1. Vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Continue this for tubes #3 through #5.

The concentration of human C4a des Arg in tubes # through #5 will be 200, 50, 12.5, 3.13, and 0.78 ng/mL respectively. See human C4a des Arg Assay Layout Sheet for dilution details. Diluted standards should be used within 60 minutes of preparation.

3. human C4a Conjugate

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

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 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 Activity wells 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 #5 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 **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 pNpp 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 human C4a 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 C4a 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:

   Average Net OD = Average Bound OD - 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 C4a des Arg for the standards. Approximate a straight line through the points. The concentration of human C4a des Arg in the unknowns can be determined by interpolation.

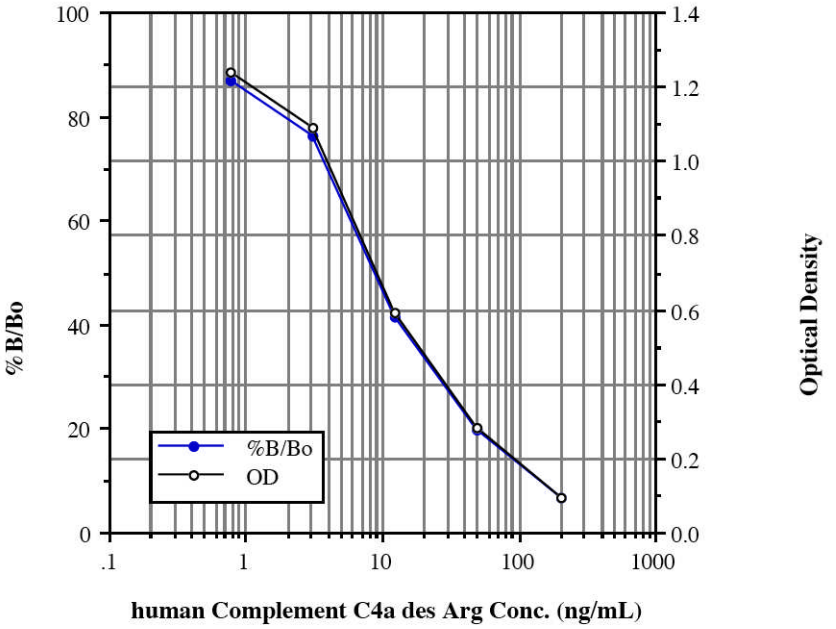
Typical Results

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

Sample	Mean OD(-Blank)	Average Net OD	Percent Bound	human C4a des Arg (ng/mL)
Blank OD	(0.122)			
TA	0.892	0.892		
NSB	-0.003	0.000		
Bo	1.424	1.427	100%	**0**
S1	0.091	0.094	6.5%	**200**
S2	0.279	0.282	19.4%	**50**
S3	0.587	0.590	40.6%	**12.5**
S4	1.086	1.089	74.9%	**3.13**
S5	1.235	1.238	85.2%	**0.78**
Unknown 1	0.187	0.190	13.1%	**73**
Unknown 2	0.620	0.623	42.9%	**12.4**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate human C4a des Arg concentrations; each user must run a standard curve for each assay.



Typical Quality Control Parameters

Total Activity Added	=	1.0x 10x10 = 101.4
%NSB	=	0.1%
%Bo/TA	=	1.5%
Quality of Fit	=	0.9960 (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

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[9].

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 #5. The detection limit was determined as the concentration of human C4a des Arg measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo =	0.902 ± 0.049 (5.4%)
Average Optical Density for Standard #5 =	0.802 ± 0.048 (5.9%)
Delta Optical Density (0-0.78 ng/mL) = 0.902-0.802 =	0.100
2 SD's of the Zero Standard = 2 x 0.049 =	0.098
Sensitivity = $\frac{0.098}{0.100}$ x 0.78 ng/mL =	**0.76 ng/mL**

Linearity

A sample containing 19.39 ng/mL human 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 C4a des Arg concentration versus measured human C4a des Arg concentration.

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

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of human C4a des Arg and running these samples multiple times (n=14) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of human C4a des Arg in multiple assays (n=6).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of human C4a des Arg determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	human C4a des Arg (ng/mL)	Intra-assay %CV	Inter-assay %CV
Low	2.2	13.9	
Medium	5.0	10.5	
High	10.8	6.7	
Low	5.13		12.5
High	10.1		11.3

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 C4a des Arg assay and the measured human C4a 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.

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%

References

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Material Safety Data Sheet (MSDS) available on our website or by fax.

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