RK-518A230421

C3a Des Arg [I-125] RIA KIT

(REF: RK-518)

For Research Use Only. Not for use in diagnostic procedures.

The [^{125}I]C3a Des Arg RIA system provides direct quantitative *in vitro* determination of human Complement C3a des Arg in human plasma. C3a des Arg can be assayed in the range of 20-500 ng/ml (1-25 ng/tube). Each kit contains materials sufficient for 150 determinations permitting the construction of one standard curve and the assay of 67 unknowns in duplicate.

Introduction

The complement system consists of a series of more than 20 proteins arranged in two separate cascades, the classical and alternative pathways. These pathways are important components of the host immune response to bacterial and viral infection. The classical pathway attenuates the humoral response, being initiated by antibody antigen complexes. The alternative pathway represents the first line of defense, being activated by a wide range of macromolecules including bacterial lipopolysaccharide, teichoic acids and immune aggregates. Activation of these cascades results in the production of complexes involved in proteolysis or cell lysis and peptides involved in opsonization, anaphylaxis and chemotaxis.

The activation product C3a (derived from C3) has potent biological activity including non-cytolytic release of histamine from mast cells and basophils, contraction of smooth muscle, and increase in capillary permeability. The activation products C4a and C5a (derived from C4 and C5) have similar properties although C4a is less potent and C5a also exhibits chemotactic activity. Release of these peptides in sufficient quantities can result in anaphylaxis in the affected individuals, hence the peptides are known as anaphylatoxins.

The three anaphylatoxins require a C-terminal arginine for full physiological activity. Removal of this arginine by a serum carboxypeptidase converts the anaphylatoxin to the physiologically inactive (C3a and C4a) or less active (C5a) des Arg form. A major problem associated with such measurements is in vitro complement activation which occurs within minutes (serum) or hours (plasma) after sample collection unless the sample is processed and stored appropriately.

Principle of method

This assay is based on the competition between unlabelled C3a and C3a des Arg and a fixed quantity of [^{125}I] –labelled C3a des Arg for a limited number of binding sites on a specific antibody. With fixed amounts of antibody and radioactive ligand, the amount of radioactive ligand bound will be inversely proportional to the concentration of added non-radioactive ligand.

The antibody bound C3a fraction is then reacted with goat anti-rabbit second antibody reagent. Separation of the antibody bound fraction is effected by centrifugation followed by decantation of the supernatant.

Measurement of the radioactivity in the pellet enables the amount of labelled C3a des Arg in the bound fraction to be calculated. The concentration of unlabelled C3a and C3a des Arg in the sample is then determined by interpolation from a standard curve. The standard curve and samples should be prepared simultaneously.

Contents of the kit

1. 1 vial TRACER (8.0 ml), ready for use after thawing, containing < 190 kBq, 5.1 µCi [^{125}I]C3a des Arg in phosphate buffered saline containing gelatin and 0.01 % sodium azide. Store at -15 ºC to -30 ºC.

2. 5 vials STANDARD (5 x 0.9 ml), ready for use after thawing, containing (A-E) 25, 10, 5, 2.5, 1 ng/ 50 µl human C3a des Arg in buffer with 0.01% sodium azide. Store at -15 ºC to -30 ºC.

3. 1 vial ANTISERUM (8.0 ml), ready for use after thawing, containing rabbit anti-C3a des Arg antiserum solution in phosphate buffered saline with porcine gelatin and 0.01% sodium azide. Store at -15 ºC to -30 ºC.

4. 1 vial ASSAY BUFFER (9.0 ml), ready for use, containing gelatin and 0.01 % sodium azide. Store at 2 ºC to 8 ºC after thawing.

5. 1 vial SECOND ANTIBODY (8.0 ml), ready for use after thawing, containing goat anti-rabbit antiserum solution in phosphate buffered saline with gelatin and 0.01% sodium azide. Store at -15 ºC to -30 ºC.

6. 1 bottle PRECIPITATING REAGENT (80 ml), ready for use, diluted in distilled water. Store at 2 ºC to 8 ºC after thawing.

Pack leaflet

Materials, tools and equipment required

Pipettes or pipetting equipment with disposable tips (50, 100, 450 and 2000 µl); disposable polypropylene, polystyrene or glass tubes (12 x 75 mm) or conical shaped test tubes capable of withstanding centrifugation speeds up to 5000 xg (for a microfuge, 1.5 ml conical tubes are acceptable); test tube rack; isotonic saline solution; vortex mixer; plastic foil; adsorbent tissue; gamma counter.

Specimen collection, storage

Both the classical and alternative pathways of complement are known to be highly unstable in vitro. As a result, sample collection and storage are critical.

Samples can be collected either in EDTA tubes or in sample collection tubes containing Futhan. During sample collection using EDTA-Futhan tubes, all operations may be carried out at room temperature. Samples are stable for up to 3 hours prior to, and up to 24 hours after separation at room temperature.

Blood samples should be separated by centrifugation at 2000 xg for 15 minutes at 4°C. Plasma should be stored in single use aliquots at -15°C to -30°C. 0.45 ml plasma is required for assay.

Note: When processing blood samples, it is important to ensure that the solid EDTA-Futhan mixture completely dissolves. This can be achieved by gently mixing the contents by inverting the tube several times. It is important that samples collected in tubes without Futhan be separated and frozen within thirty minutes. Also, frozen plasma samples collected in tubes without Futhan have limited stability.

Sample preparation

1. Label 2 sets of 12 x 75 mm disposable tubes. These tubes should be capable of withstanding centrifugation speeds of up to 5000 xg.

2. Pipette 450 µl of each sample into one of the sets of labelled tubes (sample tubes).

3. Pipette 450 µl precipitating reagent into each sample tube. (This creates a 1:1 dilution of the sample).

4. Mix each tube well.

5. Incubate for 5–30 minutes at room temperature.

6. Centrifuge at a minimum of 2500 xg for 15 minutes at 4°C. (Microcentrifuges should not be used unless they are refrigerated).

7. Transfer the supernatants to the second set of labelled tubes. Discard the first tube. The supernatant should be processed immediately, not stored for later use.

Note: If, prior to the assay, it is anticipated that sample C3a concentration will lie above the upper limit of the assay range, it is recommended that the following dilution procedure be performed.

Assays can then be performed on both diluted and undiluted samples:

Dilute by mixing the supernatant in step 7 with isotonic saline containing disodium EDTA and Futhan. The most convenient method for preparation of the diluent is to add isotonic saline to a sample collection tube specified in the previous section. The resultant solution is then used as the diluent. Investigations have shown that samples containing elevated levels of C3a des Arg can be diluted a maximum of 1:30 after addition of the precipitation reagent.

Preparation of reagents, storage

Storage: *see Contents of the kit*. At these temperatures each reagent is stable until expiry date. The actual expiry date is given on the package label and on the quality certificate.

Note: Assay components may be subjected to a maximum of two freeze-thaw cycles after initial use.

CAUTION!

Equilibrate all reagents and samples to room temperature prior to use.

Mix all reagents and samples thoroughly prior to use except for standards. Standards should be given a brief gentle mix only.

Assay procedure

(For a quick guide, refer to Table 1.)

1. Label 12x75 mm disposable tubes in duplicate for each total count (TC), non-specific binding (NSB), Bo (0 standard), standards (A-E) and each sample.
2. Pipette **50 µl** assay buffer into all tubes.
3. Vortex mix all tubes well to coat tube walls with buffer.

4. Pipette **50 µl** isotonic saline into Bo tubes and **100 µl** isotonic saline into NSB tubes.
5. Pipette **50 µl** of each standard (SA-E) directly into the bottom of appropriately labelled tubes.
6. Vortex mix each treated plasma sample (*see step 7 of Sample preparation).* Pipette **50 µl** of each sample (M_x) directly into the bottom of appropriately labelled tubes.
7. Pipette **50 µl** of assay tracer into all tubes. The TC tubes should be stoppered and set aside for counting.
8. Pipette **50 µl** of antiserum into all tubes except TC and NSB.
9. Vortex mix all tubes thoroughly. Cover the tubes, for example with plastic film, and incubate for 30 minutes at room temperature (15-30 °C).
10. Pipette **50 µl** of goat anti-rabbit second antibody into all tubes except TC. Vortex mix and incubate for 30 minutes at room temperature.
11. Add **2 ml** of isotonic saline to all tubes except TC.
12. Centrifugate all tubes together at 2000 xg for 10 minutes at 4 °C.
13. Carefully decant all tubes (except TC), discarding the supernatant. Keeping the tubes inverted, complete the removal of supernatant by standing the tubes on a pad of absorbent paper. Take care not to disturb the pellet while the tubes are inverted.
14. Count each tube for at least 60 seconds in a gamma scintillation counter (or for sufficient time to accumulate >10000 counts in the Bo tubes).
15. Calculate the C3a des Arg concentrations of the samples as described in calculation of results or use special software.

Table 1. Assay Protocol, Pipetting Guide (all volumes are in microliters)

Tubes	TC	NSB	Bo	Stan - dard	Sample
Buffer	**50**	**50**	**50**	**50**	**50**
Vortex mix					
Isotonic saline	-	**100**	**50**	-	-
Standard	-	-	-	**50**	-
Sample	-	-	-	-	**50**
Tracer	**50**	**50**	**50**	**50**	**50**
Anti-serum	-	-	**50**	**50**	**50**
Vortex mix, cover tubes and incubate for 30 minutes at room temperature (15-30 °C)					
Second antibody	-	**50**	**50**	**50**	**50**
Vortex mix and incubate for 30 minutes at room temperature					
Isotonic saline	-	**2000**	**2000**	**2000**	**2000**
Centrifuge at 2000 xg for 10 minutes (4 °C), decant tubes and blot on filter paper					
Count radioactivity (60 sec/tube)					
Calculate the results					

Calculation of results

Calculate the average count per minute (CPM) for each pair of assay tubes. Subtract the average NSB cpm from all tubes except TC. If the counter background is high, it should be subtracted from all counts. Calculate the percent Bo/TC using the following equation:

$$\text{Bo/TC(\%)} = \frac{\text{Bo (cpm)} - \text{NSB (cpm)}}{\text{TC (cpm)}} \times 100$$

The average Bo/TC(%) should be in the range 30–60%. Calculate the percent bound for each standard and sample using the following relationship:

$$\text{B/Bo(\%)} = \frac{\text{SA-E/M}_x\text{ (cpm)} - \text{NSB (cpm)}}{\text{Bo (cpm)} - \text{NSB (cpm)}} \times 100$$

A standard curve can be generated by plotting the percent B/Bo as a function of the log C3a des Arg concentration.

Plot B/Bo(%) (y-axis) against concentration (ng) C3a des Arg per tube (x-axis). The concentration (ng per tube) value of the samples can be read directly from the graph (see table 2).

The concentration ng per tube value can be converted to ng/ml by multiplying by 40 as described below:

ng/tube x2 (1:2 dilution, step 3) x 20 (50 microliters sample volume) = ng/ml

Note: If samples were further diluted, this dilution factor must be taken into account.

Note: Curve-fitting algorithms available for data reduction may be used. It should be noted that whilst results may differ by up to 5–10% depending on the algorithm used, this should not affect assay precision.

Table 2. Typical assay data

Tube	Conc. ng/50 ul	Mean counts (cpm)	B/TC (%)	B/Bo (%)
TC	-	43038	-	-
NSB	-	1020	2.4	-
Bo	-	18326	40.2	100
SE	1	15857	-	81.0
SD	2.5	12425	-	62.2
SC	5	9887	-	48.4
SB	10	7114	-	33.3
SA	25	3552	-	13.8

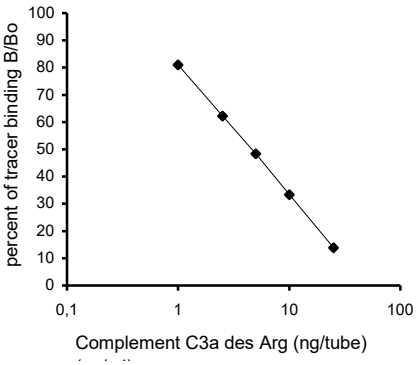


Figure 1: A typical standard curve
(*Do not use to calculate unknown samples!)*

Characterization of assay

Calibration

The assay standards are prepared from highly purified human C3a des Arg. The assay system is calibrated against a reference standard prepared from purified C3a des Arg.

Sample stability

The high sensitivity of RIA for complement activation products such as C3a means that *in vitro* complement activation in the sample must be kept to a minimum. With this in mind, the stability of samples during collection, processing and storage in tubes containing disodium EDTA and the serine protease inhibitor, Futhan, and disodium EDTA alone, has been investigated. The result are shown below:

Sample	EDTA+Futhan	EDTA only
Mean (ng/ml)	157	283
Std dev	27	104

These results were obtained from 50 blood samples collected into tubes containing EDTA+Futhan, and EDTA only. (It is recommended that individual laboratories determine levels for their own reference population).

Sensitivity

The sensitivity of the assay is defined as the C3a des Arg concentration at two standard deviations below the mean zero dose binding (n=20). This was calculated as <1 ng/50 µl (<40 ng/ml) and is below the lowest standard concentration.

Specificity

The antiserum cross-reactivities with related peptides are shown below:

Compound	Cross-reactivity (%)
C3a	100
C3a des Arg	100
C4a des Arg	0.89
C5a des Arg	<0.22

Note: The assay procedure is selective for C3a and C3a des Arg, eliminating interference from C3.

Reproducibility

The intra-assay variation (expressed as coefficient of variation) ranges from 3% to 9% depending on the C3a standard concentration. Further data on assay variation is given below.
Coefficient of variation is defined as:

$$\text{CV(\%)} = \frac{\text{Standard deviation of mean}}{\text{Mean value}} \times 100$$

Sample	Number of replicates	Mean value ng/ml	CV (%)
1	8	70	20.5
2	8	137	16.5
3	8	213	8.7

The inter-assay precision values are shown below:

Sample	Number of replicates	Mean value ng/ml	CV (%)
1	10	68	7.6
2	10	91	5.0
3	10	213	8.7

Dilution

Samples may be diluted up to 1:30 with isotonic saline containing Futhan without affecting the accuracy of determination. The diluent is prepared by adding isotonic saline to an evacuated sample collection tube containing Futhan.

Additional information

Components from various lots or from kits of different manufacturers should not be mixed or interchanged.
Do not use lipemic, hemolyzed or turbid specimens. The assay system is designed for use with sample collection tubes containing Futhan.

Precautions

Radioactivity

This product contains radioactive material. It is the responsibility of the user to ensure that local regulations or code of practice related to the handling of radioactive materials are satisfied.

Biohazard

Human blood products used in the kit have been obtained from healthy human donors. They were tested individually by using approved methods (EIA, enzyme immunoassay), and were found to be negative, for the presence of both Human Immunodeficiency Virus antibody (Anti-HIV-1) and Hepatitis B surface Antigen (HBsAg).
Care should always be taken when handling human specimens to be tested with diagnostic kits. Even if the subject has been tested, no method can offer complete assurance that Hepatitis B Virus, Human Immunodeficiency Virus (HIV-1), or other infectious agents are absent. Human blood samples should therefore be handled as *potentially infectious materials*.

Chemical hazard

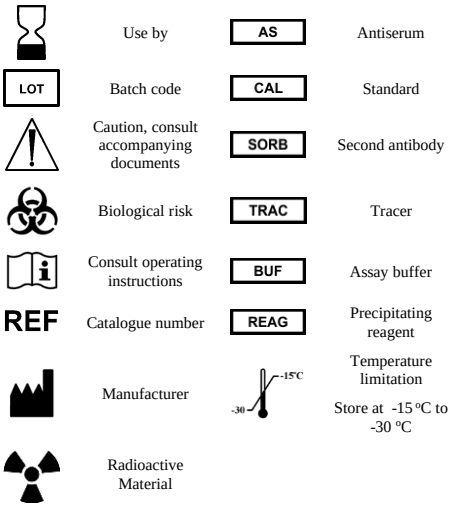
Components contain sodium azide as an antimicrobial agent. Dispose of waste by flushing with copious amount of water to avoid build-up of explosive metallic azides in copper and lead plumbing. The total azide present in each pack is 3.8 mg.

Safety data sheet

Product name:
Sodium azide
CAS No. 26628-22-8
R: 22-32 Toxic if swallowed. Contact with acids liberate very toxic gas.
S: (1/2)-28-45 (Keep locked up and out of the reach of children). After contact with skin, wash immediately with plenty of water. In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).

Composition:
Sodium azide solution.
Hazards identification:
Toxic if swallowed, inhaled, or absorbed through skin. May cause eye and skin irritation.
First aid measures:
In case of contact, immediately flush eyes or skin with copious amounts of water. If inhaled remove to fresh air. In severe cases seek medical attention.
Firefighting measures:
Dry chemical powder. Do not use water.

Accidental release:
Wear suitable protective clothing including laboratory overalls, safety glasses and gloves. Mop up spill area, place waste in a bag and hold for waste disposal. Wash spill site area after material pick-up is complete.
Handling and storage:
Wear suitable protective clothing including overalls, safety glasses and gloves. Do not get in eyes, on skin, or on clothing. Wash thoroughly after handling.
Personal protection:
See above instructions for handling and storage.
Physical and chemical properties:
Formula weight: 65.01. Density: 1.850.
Stability and reactivity:
Avoid contact with metals and acid chlorides. This yields a very toxic gas.
Toxicological information:
LD50: 27 mg/kg oral, rat
LD50: 20 mg/kg skin, rabbit
Ecological information:
Not applicable
Disposal consideration:
Up to 5 vials worth of material may be disposed of directly down the sink with water. If 6 or more vials are to be disposed of, they should pass through a chemical waste route.
Note: Inorganic azides will react with lead and copper plumbing fixtures to give explosive residues. Disposal of significant quantities of azides via such plumbing is not recommended.
Transport information:
No special considerations applicable.
Regulatory information:
The information contained in this safety data sheet is based on published sources and is believed to be correct. It should be used as a guide only. It is the responsibility of the user of this product to carry out an assessment of workplace risks, as may be required under national legislation.



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