ALPHADIA Scanlisa Anti-C1q-IgG CIC Assay

ENZYME IMMUNOASSAY FOR
CIRCULATING IMMUNE
COMPLEXES
C1q - IgG IN HUMAN SERUM

CAT # AD 196 96 TESTS

FOR IN VITRO DIAGNOSTIC USE
CONS : 2 - 8°C

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ALPHADIA C1q ELISA KIT

SUMMARY AND EXPLANATION

The harmful effects of circulating immune complexes (CIC) have been thoroughly reviewed by Theofilopoulos and Dixon and others. These CIC are not normally expressed in the serum of healthy of patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). CIC are particularly prominent in RA and SLE patients during active disease.
No single procedure appears to detect all types of CIC, but those procedures which detect CIC containing activated complement components (i.e., C1q, C3d) appear to detect clinically relevant events.
C1q ALPHADIA strip, ELISA for Circulating Immune Complexes (C1q-IgG) detects CIC containing both C1q and IgG. A positive result is indicated by the presence of CIC levels equal to or greater than 35µg/ml. A negative result is indicated by a level CIC less than 34 µg/ml. The concentration of CIC is measured as µg/ml heat aggregated human globulin (HAG) equivalents.

PRINCIPAL OF THE PROCEDURE

The procedure is a three stage test carried out on microwell strips which have been coated with monoclonal antibody specific antibody specific for the C1q component of complement (solid phase).
In the first stage, a diluted test serum is added to a test well and incubated for a specified length of time. If CIC are present in the test serum, they will attach to the solid phase anti-C1q. If CIC are not present, all serum components will be removed in the subsequent washing step.
In the second stage, monoclonal anti-human IgG conjugated to horseradish peroxidase enzyme (conjugate) is added to the test well. The conjugate will bind specifically to the human IgG (Fc) region of bound CIC. If CIC are not present in the serum, the conjugate will not be bound and will be removed in the subsequent washing step.
In the third stage, enzyme substrate is added to the test well. If bound conjugate is present the substrate will be oxidized, resulting in a colored end product. Acid is added to stop the reaction and fix the color. The color intensity is proportional to the amount of bound conjugate and therefore to the amount of CIC present in the serum specimen. The color intensity is measured with a microwell strip reader.

REAGENTS

Reagents are standardized by lot number and must not be interchanged with reagents from a kit with a different lot number.

KIT COMPONENTS (96 tests)

1 microplate (12 x 8 break-apart wells) coated with monoclonal antibody (murine) to human C1q in zip-lock foil pouch.

2 bottles Specimen diluent - ready to use

1 vial conjugate concentrate-monoclonal anti-human IgG (murine) conjugated to horseradish peroxidase; contains 0.1% Proclin 300®.

3 vials standard level I (lyophilized human serum)

3 vials standard level II(lyophilized human serum)

3 vials standard level III(lyophilized human serum)

2 vials positive control (lyophilized human serum)

2 vials negative control (lyophilized human serum)

1 bottle tetramethylbenzidine (TMB) substrate solution. Ready to use.

1 bottle wash buffer concentrate (10x).

1 bottle stop solution (1N H_2SO_4).

For in vitro diagnostic use.
Store at 2 to 8°C. Do not freeze.
Source material from which reagents of human origin were derived was found nonreactive for HBsAg and HTLV-III when tested with licensed reagents. No known test method can offer assurance that products derived from human blood will not transmit hepatitis or other infectious agents.

Caution : handle as if capable of transmitting infectious agents.

SPECIMEN COLLECTION

No special preparation of the individual is required prior to specimen collection. The whole-blood test specimen should be collected by accepted medical techniques. The specimen must be allowed to clot for two hours at room temperature (15 to 30°C) before the serum is removed.

1. Do not allow the specimen to clot at refrigerator temperature (2-8°C).
2. Do not store the clotted specimen at 2-8°C.
3. Do not use anticoagulated specimens collected as plasma and then clotted.
4. Do not heat inactivate serum specimens.
5. Do not use plasma. The serum should be separated from the clot within eight hours of collection and transferred to a plastic test tube. The test specimen can be centrifuged at 850-1500 xg for 10 minutes at ambient temperature (15 to 30°C). Mechanical serum separated devices such as ISO filter or equivalent may be used. The resulting test specimen must be completely free of cellular debris. Once the serum has been separated from the clot, it may be stored at 2-8°C for up to 24hours. If the serum specimen cannot be tested within one day of collection, the specimen must be stored at -70°C until tested. Serum specimens are stable at -70°C for at least three months. Freeze and thaw only once. Specimens may be thawed either at room temperature or for up to 5 minutes at 37°C.

MATERIAL REQUIRED BUT NOT PROVIDED

1. 1 ml,5 ml and 10 ml serological pipettes
2. 0.2 ml adjustable single-channel micropipette
3. 0.1 ml multichannel micropipette
4. 50µl to 200µl disposable pipette tips
5. Multichannel micropipette reservoir
6. Disposable glass tubes
7. Disposable plastic 25 ml tubes with cap
8. Plastic microwell strip cover or polyethylene film
9. ELISA multichannel wash bottle or automatic washing system
10. Aliquot mixer
11. 37°C humidified incubator or water bath with cover
12. Microwell strip reader capable of reading at 450 & 620 nm
13. Glass-distilled or deionized water

PRECAUTIONS

1. Disposable glass or plastic must be used for preparation of the conjugate
2. Cross contamination between reagents may invalidate the test results. The reagents should be visually inspected for evidence of bacterial or fungal contamination. Permanently labeled, dedicated multichannel micropipette reservoirs for the appropriate reagents are recommended.
3. Handle the TMB with care. Avoid contact with eyes, skin or clothing as TMB may cause irritation or an allergic skin reaction.
4. The TMB is light sensitive. Keep vial tightly closed when not in use.
5. All micropipettes must be used with care and calibrated regularly following the manufacturer's instructions.
6. The microwell strips must be thoroughly washed during the washing steps.
7. All serum specimens should be handled as if capable of transmitting disease.
8. Wash buffer, specimen diluent and distilled or deionized water must be a room temperature prior to use.
9. Diluted serum specimens and standards should be added to the microwell strips at room temperature (15 to 30°C).
10. During the second and third (last) washing procedure, disposable gloves should be worn and all reagent and washing solutions should be discarded into an appropriate container. It is recommended that household bleach be added to the waste material prior to disposal. Do not add bleach prior to washing because of possible splash back.

PREPARATION OF REAGENTS

1. **Wash buffer** (10x) : dilute the buffer 1+9 using distilled or deionized water or by pouring total contents into a graduated cylinder and add distilled or deionized water to 1L final volume. Mix thoroughly and store the 1x solution at 2-8°C.

2. **Specimen diluent** is ready to use. Do not dilute further. Allow to equilibrate to room temperature.

3. **Preparation of conjugate**
Disposable glass or plastic must be used. The conjugate concentrate must be diluted with specimen diluent just prior to use. The dilution ratio for the conjugate concentrate appears on the label. For example, if the label states dilution ration 1:100, then add 0.12 ml of conjugate concentrate to 11.86 ml specimen diluent. A volume of 12 ml is needed for twelve (12) microwell strips.
The conjugate should be prepared just prior to use and should be used within 30 minutes.

Number of strips	Ratio	Total ml	Conj conc (µl)	Specimen diluent ml
1-3	1:100	3.0 ml	30	2.97
	1:120	3.6 ml	30	3.57

	1:140	4.2 ml	30	4.17
4-6	1:100	6.0 ml	60	5.94
	1:120	7.2 ml	60	7.14
	1:140	8.4 ml	60	8.34
7-9	1:100	9.0 ml	90	8.91
	1:120	10.8 ml	90	10.71
	1:140	12.6 ml	90	12.51
10-12	1:100	12.0 ml	120	11.88
	1:120	14.4 ml	120	14.28
	1:140	16.8 ml	120	16.68

Stability at RT : 30 minutes

4. **TMB substrate**
Allow to equilibrate to room temperature. Ready to use.

5. **Reconstitution of lyophilized standards**
Reconstitute one vial of each standard (Level I,II,III) by adding 0.15 ml of glass distilled or deionized water to each vial. Gently resuspend the pellet. Allow the rehydrated standards to stand at room temperature (15 to 30°C) for 10 minutes prior to use. Do not vortex or mix vigorously. Vigorous mixing will cause denaturation of the serum CIC and result in abnormally high values. Reconstituted standards are stable for two working days. Store at 2-8°C when not in use. Each reconstituted vial of standard is sufficient for 3 wells.
Note : reconstituted standards which have been diluted 1:5 with Specimen diluent must be discarded after one working day.
6. **Reconstitution of lyophilized controls**
Reconstitute one vial of each control by adding 0.25 ml of glass distilled or deionized water to each vial. Gently resuspend the pellet. Allow the rehydrated controls to stand at room temperature (15 to 30°C) for 10 minutes prior to use. Do not vortex or mix vigorously. Vigorous mixing will cause denaturation of the serum CIC and result in abnormally high values. Reconstituted controls are stable for two working days. Store at 2-8°C when not in use. Each reconstituted vial of control is sufficient for 6 wells.
Note: reconstituted controls which have been diluted 1:5 with Specimen diluent must be discarded after one working day.

TEST PROCEDURE
1. Using disposable glass tubes, prepare 1:5 dilutions of the three reconstituted standards and the serum specimens in Specimen diluent. Mix gently by inversion. Do not vortex. Example : 0.05 ml of standard or serum specimen plus 0.2 ml of Specimen diluent (0.250 ml) is sufficient volume for duplicate testing.
2. Take the microplate from the foil pouch by cutting above the ziplock. Identify the number of microplate wells required for the assay. Remove the strips (wells) from the holder not needed for the assay and replace into the pouch and reseal with the ziplock provided, leaving the desiccant packs inside. When stored at 2-8°C, resealed pouches will retain their reactivity. A pouch which has not been resealed or refrigerated after opening should be discarded.
3. Wash the microwell strips, including reagent blank well (RB), three times with 1x wash buffer. After the last wash, tap microwell strips firmly on absorbent paper to remove excess wash buffer. Use the microwell strips immediately after washing. Do not allow wells in the microwell strips to dry.
4. The upper left well (RB) on each frame is reserved for blanking the microwell strip reader. Refer to the manufacturer's instructions for blanking your reader.
5. Mark the microwell strip holder to indicate location of reagent blank, standards and serum specimens. Standards must be run in duplicate with each run.
Note: The standards and the serum specimens in steps 7-9 must be added to the microwell strips within 10 minutes. Pipette tips should be changed for each different standard and test specimen. Add 100µl of each dilution standard and controls, in duplicate, to the appropriate wells.
6. Add 100µl of Specimen diluent to the reagent blank well.
7. Add 100µl of each diluted serum specimen, in duplicate, to the appropriate wells.
8. Cover the microwell strip holder with a cover and incubate in a moist environment at 32 to 37°C for 60 minutes.

- a. In a 37°C water bath: The covered microwell strips should not be floated on the water, but rested on a level support in the water bath, close to the surface of the water. Keep bath covered during incubation. Air temperature at level of microwell strips shoul be 32 to 37°C.
- b. In a 37°C air incubator : Place a container with a cover inside the incubator. Leave this container inside the incubator to be sure the temperature inside is 37°C. Maintain the moisture by placing damp paper towels in the bottom of the container. Place microwell strip holder inside the container and keep the cover on for the incubation period.

9. Prepare conjugate. Refer to preparation reagents.
10. Wash the microwell strips, including blanking well(s), three times with 1x wash buffer. The washing procedure must be thorough. If an ELISA multichannel wash bottle is used:
- a. Empty wells by vigorously shaking out contents.
- b. Invert wash bottle and thoroughly flush wells with wash buffer by squeezing the bottle.
- c. Remove wash buffer by vigorously shaking out contents.
- d. Repeat steps 10b and 10c twice.
- e. After the final wash, tap the inverted holder firmly on a clean paper towel to remove excess wash buffer.
- f. Proceed immediately to the next step.

Note: Do not allow wells to dry during the test. Drying of the wells may result in falsely high absorbance values.
11. Using a multichannel micropipette, add 100µl of conjugate to all wells, including reagent blank well
12. Incubate the microwell strips (uncovered) at room temperature in the dark for 60 minutes.
13. Wash the microwell strips as described in step n° 10. Proceed immediately to the next step.
14. Using a multichannel micropipette, add 100 µl of substrate to all wells, including the reagent blank well.
15. Incubate the microwell strips (uncovered) at room temperature in the dark for 30 minutes.
16. Using a multichannel micropipette, forcibly eject 100 µl of 1N sulfuric acid into each well, including the reagent blank well. It is important that the sulfuric acid is spread quickly and uniformly throughout the well to completely inactive the enzyme. Results can be read up to one hour after the sulfuric acid is added if the microwell strips are stored in the dark.
17. Set the microwell strip reader at a wavelength of 450 nm and blank the reader according to the manufacturer's instructions by using the reagent blank well containing substrate and acid.
18. Measure the color intensity in all microwell strips.

INTERPRETATION OF RESULTS

Calculate the mean absorbance value of the duplicate standards (levels I,II,III) and controls. Duplicates should be within 20 percent of the mean.

Preparation of the standard curve and determination of the Immune Complex concentration (µg/ml HAG equivalents)

1. Calculation method

Calculations can be made on a variety of calculators supplied with linear regression programs. Follow the manufacturer's instructions for use of these programs to establish the standard curve and the immune complex concentration (µg/ml HAG equivalents) of each serum specimen.

In order for the assay to be valid, the following criteria must be met :

a. Slope	0.005-0.009
b.Y-intercept	less than 0.450
c. Correlation Coefficient (r^2)	greater than 0.950

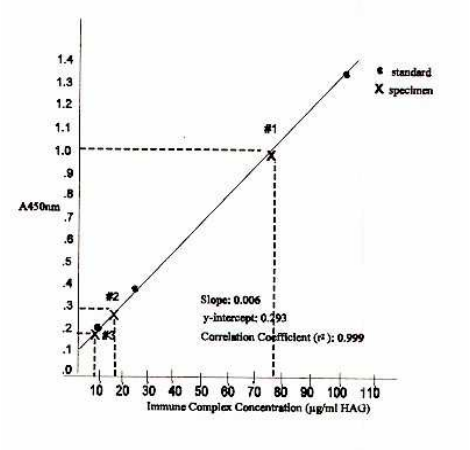
2. Graph Method

a. Plot the mean absorbance values for standards (level I,II,III) on the ordinate against the corresponding µg/ml HAG on the abscissa, using linear graph paper. Refer to vial labels of standards for assigned values expressed as µg/ml.
b. Draw a straight line for the best fit of the plotted points.
c. Locate the point corresponding to the average absorbance value of each sera specimen and determine the corresponding µg/ml HAG equivalents.
d. Record the immune complex concentration for each serum as µg/ml HAG equivalents.

3. Illustration of standard curve

A representative standard curve is shown in Figure 1. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed (up to twelve). Refer to each vial label of standard (level I,II,III) for assigned values expressed as µg/ml.

Figure 1. Standard curve



	Absorbance value	Immune Complex Concentration (µg/ml HAG equivalents)
Standard level I	0.420	18
Standard level II	0.584	48
Standard level III	1.192	140
Specimen 1	1.135	128
Specimen 2	0.571	43
Specimen 3	0.314	3.3

Criteria for interpretation of results

1. **Negative results**
Results from 0 to 34 µg/ml HAG equivalents can be considered negative for significant levels of CIC.
2. **Positive results**
Results of 35 µg/ml HAG equivalents or greater can be interpreted as positive for significant levels of CIC.

LIMITATIONS OF PROCEDURES

1. Falsely elevated results will be obtained if :
   a. Plasma is used instead of serum.
   b. Specimens are collected as plasma in anticoagulants and subsequently defibrinated.
   c. Serum specimens have been heat inactivated.
   d. Specimens are improperly handled or stored.
2. Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results.
3. Adherence to the humidity, temperature and time periods for incubation is essential for accurate results.
4. Bubbles or excess reagent in the wells may result in cross contamination of the wells when a microwell cover is used.
5. Bacterial or fungal contamination of either serum specimens or reagents or cross contamination between reagents may cause erroneous results.
6. A small percentage of the population will have values close to the statistically derived upper limit of normal.These results should be interpreted as borderline. For these individuals, retesting is recommended.
7. If another manufacturer's test is used to determine CIC, the laboratory should establish normal ranges for the test being used.
8. CIC formation may be associated with pathological conditions other than RA and SLE.

EXPECTED VALUES

Normal
Approximately 3% of healthy, asymptomatic individuals may have CIC concentrations greater than 34 µg/ml HAG.
A total of 488 serum samples from healthy, asymptomatic individuals were assayed. The mean value ± SD was 3.7 ± 15.3 µg/ml equivalents. Fourteen (2.9%) of these samples had immune complex concentrations greater than or equal to 35 µg/ml HAG.

Frequency distribution (%) of CIC values for 488 healthy asymptomatic individuals

µg/ml HAG	0-9	10-19	20-29	30-34	35-39	40-49	>149
Serum specimens %	89.9	5.1	2.0	0.4	0.6	1.2	1.0

Rheumatoid Arthritis (RA)
The following data were obtained from testing pedigreed serum samples collected serially from patients with rheumatoid arthritis :

CIC concentrations and frequency distribution in 151 sera from 18 patients with Rheumatoid Arthritis

CIC concentrations

Disease activity	n	Mean ±SD (µg/ml HAG)	% > 34µg/ml HAG
Remission	42	11.4 ± 3.7	0
Active	109	104.5 ± 63.4	86.42

Frequency distribution of CIC concentrations

µg/ml HAG	0-9	10-19	20-34	35-49	50-99	100-149	> 149
Remission %	81	9.5	9.5	0	0	0	0
Active %	6.6	0.9	6.4	6.4	33.9	20.1	25.7

The following data were obtained from testing individual serum samples obtained from clinically diagnosed rheumatoid arthritis patients. These sera were not classified by disease activity, i.e., remission or active.

CIC concentrations and frequency distribution in 50 sera from 50 patients with Rheumatoid Arthritis

1. CIC concentrations

	n	Mean ± SD (µg/ml HAG)	% > 34 µg/ml HAG
RA sera	50	137 ± 261	54

2. Frequency distribution of CIC concentrations

µg/ml HAG	0-9	10-19	20-34	35-49	50-99	100-149	>149
RA sera %	24	6	16	10	14	10	20

Systemic Lupus Erythematosus (SLE)

The following data were obtained from testing pedigreed serum samples collected serially from patients with systemic lupus erythematosus :

CIC concentrations and frequency distribution 25 sera 15 patients with Systemic Lupus Erythematosus

1. CIC concentrations

Disease activity	n	Mean ± SD µg/ml HAG	% >34 µg/ml HAG
Inactive	5	20.4 ± 21.4	20
Active	20	121.7 ± 58.6	100

2. Frequency distribution of CIC concentrations

µg/ml HAG	0-9	10-19	20-34	35-49	50-99	100-149	>149
Inactive %	40	40	0	0	20	0	0
Active %	0	0	0	5	40	30	25

The following data were obtained from testing individual serum samples obtained from clinically diagnosed systemic lupus erythematosus patients. These sera have not been classif by disease activity, i.e., inactive or active.

CIC concentrations and frequency distribution in 50 sera from 50 patients with SLE

1. CIC concentrations

	n	Mean ± SD (µg/ml HAG)	% > 34 µg/ml HAG
SLE sera	50	77.48 ± 96.5	60

2. Frequency distribution of CIC concentrations

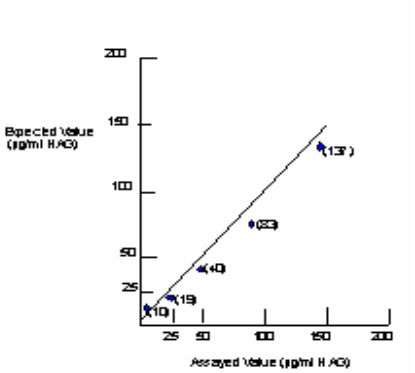
µg/ml HAG	0-9	10-19	20-34	35-49	50-99	100-149	>149
SLE sera %	28	2	10	12	14	18	16

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy
A World Health Organization (WHO) standard for CIC assays containing 800µg of heat aggregated IgG per ml was used to prepare standards in fresh frozen human serum ranging in concentrations from 10 to 150 µg/ml. The samples were coded and assayed as unknowns using C1q ALPHADIA strip ELISA for Circulating Immune Complexes (C1q-IgG). The assayed values are shown plotted on the abscissa against the expected values plotted on the ordinate in Figure 2. The actual assay values appear in parentheses. Each point represents the mean of triplicate absorbance values. The response was assumed linear over the entire test range.

Assay of WHO standard for immune complex assay using C1q ALPHADIA strip ELISA for CIC (C1Q-IgG).

Figure 2



Linearity
C1q ALPHADIA strips ELISA for CIC (C1q-IgG) is linear to 200µg/ml HAG equivalents as shown in Figure 2 above.

Precision
Eighty replicates of single specimen were assayed on each of three microtitration plates on two consecutive days. The mean value of the specimen on day 1 was 68 µg/ml HAG equivalents and the coefficient of variation of the absorbance for each

of the plates was 7.6%, 7.7% and 4.9%. The mean value of the specimen run on day 2 was 41 µg/ml HAG equivalents and the coefficient of variation of the absorbance values for each of the plates was 8.8%, 5.2% and 7.0%. The average coefficient of variation of all six plates was 6.9%.
Twenty serum specimens were tested in duplicate on three different plates on four consecutive days and converted to µg/ml HAG equivalents. The average coefficient of variation between the plates was 8.6%. The coefficient of variation on each of the four days was 5.4%, 3.2%, 8.1% and 2.4%. The range of values for the 20 specimens was from 13 to greater than 300 µg/ml HAG equivalents (x=114.8 ± 62.8).

Sensitivity
The sensitivity of C1q ALPHADIA strips ELISA for CIC (C1q-IgG) is 10 µg/ml HAG equivalents.

Specificity
Six serum panels totaling 488 specimens were obtained from healthy, asymptomatic individuals and assayed in two laboratories for CIC.

Panel 1:Five of 98 (5.1%) of the sera assayed had values greater than 34 µg/ml HAG.
Panel 2: None of 94 (0.0%) of the sera assayed had a value greater than 34 µg/ml HAG.
Panel 3: Three of 49 (6.4%) of the sera assayed had a values greater than 34 µg/ml HAG.
Panel 4: One of 60 (1.7%) of the sera assayed had a value greater than 34 µg/ml HAG.
Panel 5: Five of 100 (5.0%) of the sera assayed had a values greater than 34 µg/ml HAG.
Panel 6: None of 87 (0.0%) of the sera assayed had a value greater than 34 µg/ml HAG.

The total number of sera with values greater than 34 µg/ml was 2.9%, resulting in a specificity of 97.1%.

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