CIC C3d ELISA
Direct immunoenzymatic determination of Circulating Immune Complex C3d (CIC C3d) in human serum or plasma

**INTENDED USE**
Eagle Biosciences CIC C3d ELISA Assay Kit is an immunoenzymatic colorimetric method for quantitative determination of Circulating Immune Complex C3d (CIC C3d) concentration in human serum or plasma. CIC C3d ELISA kit is intended for research use only not to be used in diagnostic procedures.

1. CLINICAL SIGNIFICANCE
The importance of the immunocomplex (CIC) and their relation with several diseases have been object of investigations for many years. The establishment of immunocomplex is a normal protecting process of the immune system. The circulating immunocomplex are removed from the circulation by means of various cellular, biochemical and enzymatic processes. Key of elimination of many CIC is the activation of the classic way of the complement. In some diseases, of difficult understanding, the immunocomplex can begin the damaging of tissue and organs. In this case the activation of the complement can lead to the anafilotoxin production, stimulation of leukocyte and activation of macrophage and other cells. In some cases of glomerulonephritis, in which the immunocomplex fix to the cellular membranes, it has the destruction of the tissue.

Circulating immunocomplexes (CIC) are present in many individuals affections from systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA), especially in those affections from vasculitis complications. There are many tests for the determination of CIC, included the test of precipitation with PEG, radial immunodiffusion, and cellular tests like the test of Ray cell. Does not exist one procedure to determinate all types of immunocomplex; in commerce some test to determinate fragments of the complex are available (Es. C1q and C3d), that have an important diagnostic meaning.

2. PRINCIPLE
In the CIC C3d ELISA Assay Kit, the C3d-fixing circulating immune complexes (CIC) are first blocked by the anti-C3d immobilized on the microplate. During this phase, the immunocomplex binds to the antibodies anti C3d coated on the microplate. The microplate is washed to remove the unbound serum protein.

In the second phase of the CIC C3d ELISA Assay Kit, the anti human IgG antibodies conjugated with peroxidase are added; they bind to the immunocomplex fixed on the microplate. The washing solution removes the unbound conjugate. In the third phase, the TMB Substrate is added, and reacts with the conjugate fixed on the microplate. The quantity of CIC IgG complex is proportional to the color intensity read at 450 nm wavelengths. The immunocomplex concentration in the sample is calculated through a calibration curve. "Heat aggregate human gamma globulin per mL" (μgEq/mL) is the unit of measure of the results.

3. REAGENTS, MATERIALS AND INSTRUMENTATION

3.1. Reagents and materials supplied in the kit
1. CIC C3d Calibrator (3 vials, 1.5 mL each)
   - CAL0  REF DCE002/1706-0
   - CAL1  REF DCE002/1707-0
   - CAL2  REF DCE002/1708-0
2. Controls (2 vials, 1.5 mL each)
   - Negative Control  REF DCE045/1701-0
   - Positive Control  REF DCE045/1702-0
3. Incubation Buffer (1 vial, 50 mL)
   - Phosphate buffer 74 mM pH 7.4; BSA 1 g/L  REF DCE008-0
4. Conjugate (1 vial, 0.5 mL)
   - Anti human IgG antibodies conjugated with horseradish peroxidase (HRP)  REF DCE002/1702-0
5. Conjugate Buffer (1 vial, 20 mL)
   - Phosphate buffer 74 mM pH 7.4; BSA 1 g/L  REF DCE009-0
6. Coated Microplate (1 breakable microplate)
   - Anti C3d antibodies coated on microplate  REF DCE002/1703-0
7. 10X Conc. Wash solution (2 vials, 50 mL ciascuno)
   - NaCl 160 g/L; tween-20 10 g/L; Phosphate buffer 0.2M pH 7.4  REF DCE054-0
8. TMB Substrate (1 vial, 15 mL)
   - H2O2-TMB (0.26 g/L) (avoid any skin contact)  REF DCE004-0
9. **Stop Solution** (1 vial, 15 mL)
   Sulphuric Acid 0.15 mol/L (avoid any skin contact) REF DCE005-0

3.2. **Reagents necessary not supplied**
Distilled water.

3.3. **Auxiliary materials and instrumentation**
Automatic dispenser.
Microplates reader (450 nm)

**Note**
All reagents and the microplate should be stored at 2-8°C in the dark and used within the expiry date written on the package.
Keep the microplate at room temperature for few minutes prior to removing a number of wells necessary for the assay.
Place the unused microwell strips into the storage bag and reseal the bag with tape.

4. **WARNINGS**
- This CIC C3d ELISA Assay Kit is intended for research use by professional persons only. Not for internal or external use in Humans or Animals.
- Use appropriate personal protective equipment while working with the reagents provided.
- Follow Good Laboratory Practice (GLP) for handling blood products.
- All human source material used in the preparation of the reagents has been tested and found negative for antibody to HIV 1&2, HbsAg, and HCV. No test method however can offer complete assurance that HIV, HBV, HCV or other infectious agents are absent. Therefore, Calibrators and Controls should be handled in the same manner as potentially infectious material.
- Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy and the bovine protein has been obtained from countries not infected by BSE, but these materials should be handled as potentially infectious.
- Some reagents of the CIC C3d ELISA Assay Kit contain small amounts of Proclin 300® as preservatives. Avoid the contact with skin or mucosa.
- The TMB Substrate contains an irritant, which may be harmful if inhaled, ingested or absorbed through the skin. To prevent injury, avoid inhalation, ingestion or contact with skin and eyes.
- The Stop Solution consists of a diluted sulphuric acid solution. Sulphuric acid is poisonous and corrosive and can be toxic if ingested. To prevent chemical burns, avoid contact with skin and eyes.
- Avoid the exposure of reagent TMB/H₂O₂ to directed sunlight, metals or oxidants. Do not freeze the solution.

5. **PRECAUTIONS**
- Please adhere strictly to the sequence of pipetting steps provided in this protocol. The performance data represented here were obtained using specific reagents listed in this Instruction For Use.
- All reagents of the CIC C3d ELISA Assay Kit should be stored refrigerated at 2-8°C in their original container. Any exceptions are clearly indicated. The reagents are stable until the expiry date when stored and handled as indicated.
- Allow all CIC C3d ELISA Assay Kit components and specimens to reach room temperature (22-28°C) and mix well prior to use.
- Do not interchange CIC C3d ELISA Assay Kit components from different lots. The expiry date printed on box and vials labels must be observed. Do not use any kit component beyond their expiry date.
- **WARNING:** the conjugate reagent is designed to ensure maximum dose sensitivity and may be contaminated by external agents if not used properly; therefore, it is recommended to use disposable consumables (tips, bottles, trays, etc.). For divided doses, take the exact amount of conjugate needed and do not re-introduce any waste product into the original bottle. In addition, for doses dispensed with the aid of automatic and semi-automatic devices, before using the conjugate, it is advisable to clean the fluid handling system, ensuring that the procedures of washing, deproteinization and decontamination are effective in avoiding contamination of the conjugate; this procedure is highly recommended when the kit is processed using analyzers which are not equipped with disposable tips. For this purpose, Diametra supplies a separate decontamination reagent for cleaning needles.
- If you use automated equipment, the user has the responsibility to make sure that the kit has been appropriately tested.
- The incomplete or inaccurate liquid removal from the wells could influence the assay precision and/or increase the background.
- It is important that the time of reaction in each well is held constant for reproducible results. Pipetting of samples should not extend beyond ten minutes to avoid assay drift. If more than 10 minutes are needed, follow the same order of dispensation. If more than one plate is used, it is recommended to repeat the dose response curve in each plate.
- Addition of the TMB Substrate solution initiates a kinetic reaction, which is terminated by the addition of the Stop Solution. Therefore, the TMB Substrate and the Stop Solution should be added in the same sequence to eliminate any time deviation during the reaction.
- Observe the guidelines for performing quality control in medical laboratories by assaying controls and/or pooled sera.
- Maximum precision is required for reconstitution and dispensation of the reagents.
- Samples microbiologically contaminated, highly lipemic or haemolysed should not be used in the assay.
- Plate readers measure vertically. Do not touch the bottom of the wells.
6. PROCEDURE

6.1. Preparation of Calibrators
The Calibrators are ready to use and have the following concentrations:

<table>
<thead>
<tr>
<th>µgEq/mL</th>
<th>C₀</th>
<th>C₁</th>
<th>C₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>16</td>
<td>64</td>
</tr>
</tbody>
</table>

Once opened the Calibrators are stable 6 months at 2-8°C.

6.2. Preparation of Diluted Conjugate
Dilute the concentrated Conjugate (reagent 4) 1:100 with Conjugate buffer (reagent 5). The quantity of diluted Conjugate is proportional to the number of the assays. Mix well and avoid foaming. Stable for 3 hours at room temperature (22±28°C).

6.3. Preparation of Wash Solution
Dilute the content of the vial "10X Conc. Wash Solution" with distilled water to a final volume of 500 mL prior to use. For smaller volumes respect the 1:10 dilution ratio. The diluted wash solution is stable for 30 days at 2-8°C.

In concentrated wash solution it is possible to observe the presence of crystals, in this case mix at room temperature until complete dissolution of crystals, for greater accuracy dilute the whole bottle of concentrated wash solution to 500 mL taking care also to transfer crystals, then mix until crystals are completely dissolved.

6.4. Preparation of the Sample
The CIC assay can be performed in human serum or plasma. Samples, which are not immediately processed (within 24 h), should be stored at -20°C. Samples should not be thawed more than once.

Pipette in a test tube:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Calibrator</th>
<th>Sample/ Controls</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₀-C₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluted</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Incubate 30 minutes at 37°C. Remove the contents from each well, wash the wells three times with 300 µL diluted wash solution.

Diluted Conjugate 100 µL 100 µL

Incubate 30 minutes at 37°C. Remove the contents from each well, wash the wells three times with 300 µL diluted wash solution.

TMB Substrate 100 µL 100 µL 100 µL

Incubate 15 minutes in the dark at room temperature (22-28°C).

Stop Solution 100 µL 100 µL 100 µL

Shake the microplate gently. Read Absorbance (E) at 450 nm against Blank within 5 minutes.

6.5. Procedure
- Allow all reagents to reach room temperature (22-28°C) for at least 30 minutes.
- Unused coated microwell strips should be released securely in the foil pouch containing desiccant and stored at 2-8°C.
- To avoid potential microbial and/or chemical contamination, unused reagents should never be transferred into the original vials.
- As it is necessary to perform the determination in duplicate in order to improve accuracy of the test results, prepare two wells for each point of the calibration curve (C₀-C₂), two for each Control, two for each sample, one for Blank.

7. QUALITY CONTROL
Each laboratory should assay controls at normal, high and low levels range of Cortisol for monitoring assay performance. These controls should be treated as unknowns and values determined in every test procedure performed. Quality control charts should be maintained to follow the performance of the supplied reagents. Pertinent statistical methods should be employed to ascertain trends. The individual laboratory should set acceptable assay performance limits. Other parameters that should be monitored include the 80, 50 and 20% intercepts of the calibration curve for run-to-run reproducibility. In addition, maximum absorbance should be consistent with past experience. Significant deviation from established performance can indicate unnoticed change in experimental conditions or degradation of kit reagents. Fresh reagents should be used to determine the reason for the variations.

8. RESULTS

8.1. Mean Absorbance
Calculate the mean of the absorbance (Em) for each point of the calibration curve and of each sample.

8.2. Calibration curve
Plot the mean value of absorbance (Em) of the Calibrators (C₀-C₂) against concentration. Draw the best-fit curve through the plotted points. (es: Four Parameter Logistic).
8.3. Calculation of Results
Interpolate the values of the samples on the calibration curve to obtain the corresponding values of the concentrations expressed in μgEq/mL.

9. REFERENCE VALUES

<table>
<thead>
<tr>
<th></th>
<th>μgEq/mL of aggregates IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Sample</td>
<td>&lt;16</td>
</tr>
<tr>
<td>Uncertain Sample</td>
<td>between 16 and 18</td>
</tr>
<tr>
<td>Positive Sample</td>
<td>&gt;18</td>
</tr>
</tbody>
</table>

Please pay attention to the fact that the determination of a range of expected values for a “normal” population in a given method is dependent on many factors, such as specificity and sensitivity of the method used and type of population under investigation. Therefore each laboratory should consider the range given by the Manufacturer as a general indication and produce their own range of expected values based on the indigenous population where the laboratory works.

10. PERFORMANCE AND CHARACTERISTICS

10.1. Precision

10.1.1. Intra Assay Variation
Within run variation was determined by replicate determination (16x) of two different control sera in one assay. The within assay variability is 6.1%.

10.1.2. Inter Assay Variation
Between run variations was determined by replicate measurements of three different control sera in 2 different lots. The between assay variability is 13.9%.

10.2. Accuracy
The recovery of 12.5 – 25 – 50 μgEq/mL of CIC C3d added to “serum-free” sample gave an average value (±SD) of 99.84% ± 5.07% with reference to the original concentrations.

10.3. Sensitivity
The lowest detectable concentration of CIC C3d that can be distinguished from the zero Calibrator is 0.60 μgEq/mL at the 95 % confidence limit.

11. WASTE MANAGEMENT
Reagents must be disposed off in accordance with local regulations.

BIBLIOGRAPHY
ERROR POSSIBLE CAUSES / SUGGESTIONS

No colorimetric reaction
- no conjugate pipetted reaction after addition
- contamination of conjugates and/or of substrate
- errors in performing the assay procedure (e.g. accidental pipetting of reagents in a wrong sequence or from the wrong vial, etc.)

Too low reaction (too low ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too short, incubation temperature too low

Too high reaction (too high ODs)
- incorrect conjugate (e.g. not from original kit)
- incubation time too long, incubation temperature too high
- water quality for wash buffer insufficient (low grade of deionization)
- insufficient washing (conjugates not properly removed)

Unexplainable outliers
- contamination of pipettes, tips or containers
- insufficient washing (conjugates not properly removed) too high within run
- reagents and/or strips not pre-warmed to CV% Room Temperature prior to use
- plate washer is not washing correctly (suggestion: clean washer head)
- too high between-run - incubation conditions not constant (time, CV % temperature)
- controls and samples not dispensed at the same time (with the same intervals) (check pipetting order)
- person-related variation