CIC-C1q ELISA (IgG)
Test instruction

ORDER NO.	Circulating Immune Complexes (CIC-C1q)	SUBSTRATE	FORMAT
EA 1818-9601 G		C1q-coated microplate wells	96 x 01 (96)

Indication: The ELISA test kit provides a semiquantitative or quantitative in vitro assay against C1q-binding-circulating immune complexes (CIC) containing IgG in human serum or plasma for the diagnosis of rheumatic, autoimmune and infectious diseases, allergy, haematological and neoplastic disorders.

Application: Increased levels of circulating immune complexes can be detected in patients with different autoimmune, microbial and neoplastic diseases. Immune complexes are produced as a consequence of the body's immune response. Investigation of these complexes can therefore be useful for assessing the disease activity, for therapy control and for establishing a prognosis. Moreover, circulating immune complexes can become pathogenetically active through complement activation.

Principle of the test: The test kit contains microplate strips, each with 8 break-off reagent wells coated with complement fragment C1q. In the first reaction step, diluted patient samples are incubated in the wells. If samples are positive, circulating immune complexes bind to the C1q via the Fc fragment of the immunoglobulins. To detect the bound immune complexes, a second incubation is carried out using an enzyme-labelled anti-human IgG (enzyme conjugate) catalysing a colour reaction. The intensity of the colour formed is proportional to the concentration of circulating immune complexes against C1q.

Contents of the test kit:

Component	Colour	Format	Symbol
1. Microplate wells coated with C1q 12 microplate strips each containing 8 individual break-off wells in a frame, ready for use	---	12 x 8	STRIPS
2. Calibrator 1 200 RU/ml (CIC-C1q, human), lyophilised	colourless	1 x 2.0 ml	CAL 1
3. Calibrator 2 20 RU/ml (CIC-C1q, human), lyophilised	colourless	1 x 2.0 ml	CAL 2
4. Calibrator 3 2 RU/ml (CIC-C1q, human), lyophilised	colourless	1 x 2.0 ml	CAL 3
5. Positive control (CIC-C1q, human), lyophilised	colourless	1 x 2.0 ml	POS CONTROL
6. Negative control (CIC-C1q, human), lyophilised	colourless	1 x 2.0 ml	NEG CONTROL
7. Enzyme conjugate peroxidase-labelled anti-human IgG (rabbit), ready for use	light green	1 x 12 ml	CONJUGATE
8. Sample buffer ready for use	bluish	1 x 100 ml	SAMPLE BUFFER
9. Wash buffer 10x concentrated	colourless	1 x 100 ml	WASH BUFFER 10x
10. Chromogen/substrate solution TMB/H_2O_2, ready for use	colourless	1 x 12 ml	SUBSTRATE
11. Stop solution 0.5 M sulphuric acid, ready for use	colourless	1 x 12 ml	STOP SOLUTION
12. Plastic foil	---	2 pieces	
13. Test instruction	---	1 booklet	
14. Quality control certificate	---	1 protocol	

LOT Lot description
IVD In vitro diagnostic medical device
CE
Storage temperature
Unopened usable until

Preparation and stability of the reagents

Note: All reagents must be brought to room temperature (+18°C to +25°C) approx. 30 minutes before use. After first use, the reagents are stable until the indicated expiry date if stored at +2°C to +8°C and protected from contamination, unless stated otherwise below.

- **Coated wells:** Ready for use. Tear open the resealable protective wrapping of the microplate at the recesses above the grip seam. Do not open until the microplate has reached room temperature to prevent the individual strips from moistening. Immediately replace the remaining wells of a partly used microplate in the protective wrapping and tightly seal with the integrated grip seam (Do not remove the desiccant bag).
  Once the protective wrapping has been opened for the first time, the wells coated with C1q can be stored in a dry place and at a temperature between +2°C and +8°C for 4 months.
- **Calibrators and controls:** Lyophilised. Reconstitute the content of each vial with 2 ml deionised or distilled water. The reconstituted calibrators and controls must be mixed thoroughly before use.
  The reconstituted calibrators and controls are stable at +18°C to +25°C for up to 4 hours. If the reconstituted calibrators and controls are to be stored for a longer period of time, they should be kept aliquotated at -20°C or lower until expiry date. Repeated freeze-thaw cycles of calibrators and controls should be avoided.
- **Enzyme conjugate:** Ready for use. The enzyme conjugate must be mixed thoroughly before use.
- **Sample buffer:** Ready for use.
- **Wash buffer:** The wash buffer is a 10x concentrate. If crystallisation occurs in the concentrated buffer, warm it to +37°C and mix well before diluting. The quantity required should be removed from the bottle using a clean pipette and diluted with deionised or distilled water (1 part reagent plus 9 parts distilled water).
  For example: For 1 microplate strip, 5 ml concentrate plus 45 ml water.
  The working strength wash buffer is stable for 4 weeks when stored at +2°C to +8°C and handled properly.
- **Chromogen/substrate solution:** Ready for use. Close the bottle immediately after use, as the contents are sensitive to light. The chromogen/substrate solution must be clear on use. Do not use the solution if it is blue coloured.
- **Stop solution:** Ready for use.

Storage and stability: The test kit has to be stored at a temperature between +2°C to +8°C. Do not freeze. Unopened, all test kit components are stable until the indicated expiry date.

Waste disposal: Patient samples, calibrators, controls and incubated microplate strips should be handled as infectious waste. All reagents must be disposed of in accordance with local disposal regulations.

Warning: The calibrators and controls of human origin have tested negative for HBsAg, anti-HCV, anti-HIV-1 and anti-HIV-2. Nonetheless, all materials should be treated as being a potential infection hazard and should be handled with care. Some of the reagents contain the agent sodium azide in a non-declarable concentration. Avoid skin contact.

Preparation and stability of the patient samples

Samples: Human serum or EDTA plasma.

Stability: Patient samples to be investigated can generally be stored at +2°C to +8°C for up to 14 days. Diluted samples should be incubated within one working day.

Sample dilution: Patient samples are diluted **1:51** in sample buffer. For example: dilute 10 µl sample in 0.5 ml sample buffer and mix well by vortexing (sample pipettes are not suitable for mixing).

NOTE: The calibrators and controls are prediluted and ready for use after reconstitution, do not dilute them.

Incubation

For **semiquantative analysis** incubate **calibrator 2** along with the positive and negative controls and patient samples. For **quantitative analysis** incubate **calibrators 1, 2 and 3** along with the positive and negative controls and patient samples.

(Partly) manual test performance

Sample incubation: (1st step)
Transfer 100 µl of the calibrators, positive and negative controls or diluted patient samples into the individual microplate wells according to the pipetting protocol. Cover the wells and incubate for **30 minutes** at room temperature (+18°C to +25°C) on a **microplate shaker** (**600 rpm**, horizontal rotary shaker with a shaking amplitude of approx. 3 mm, e.g. microplate shaker TPM 4 from Sarstedt or Compact Digital Microplate Shaker from Thermo Scientific).*

Washing:
Manual: Remove the plastic foil. Empty the wells and subsequently wash 3 times using 350 µl of working strength wash buffer for each wash.
Automatic: Remove the plastic foil and wash the reagent wells 3 times with 450 µl of working strength wash buffer (program setting: e.g. TECAN Columbus Washer "Overflow Mode").

Leave the wash buffer in each well for 30 to 60 seconds per washing cycle, then empty the wells. After washing (manual and automated tests), thoroughly dispose of all liquid from the microplate by tapping it on absorbent paper with the openings facing downwards to remove all residual wash buffer.

Note: Residual liquid (> 10 µl) in the reagent wells after washing can interfere with the substrate and lead to false low extinction values. Insufficient washing (e.g. less than 3 wash cycles, too small wash buffer volumes, or too short residence times) can lead to false high extinction values.
Free positions on the microplate strip should be filled with blank wells of the same plate format as that of the parameter to be investigated.

Conjugate incubation: (2nd step)
Pipette 100 µl of enzyme conjugate (peroxidase-labelled anti-human IgG) into each of the microplate wells. Incubate for **30 minutes** at room temperature (+18°C to +25°C) on a **microplate shaker** (**600 rpm**, horizontal rotary shaker with a shaking amplitude of approx. 3 mm, e.g. microplate shaker TPM 4 from Sarstedt or Compact Digital Microplate Shaker from Thermo Scientific).

Washing:
Empty the wells. Wash as described above.

Substrate incubation: (3rd step)
Pipette 100 µl of chromogen/substrate solution into each of the microplate wells. Incubate for **15 minutes** at room temperature (+18°C to +25°C) (protect from direct sunlight).

Stopping:
Pipette 100 µl of stop solution into each of the microplate wells in the same order and at the same speed as the chromogen/substrate solution was introduced.

Measurement:
Photometric measurement of the colour intensity should be made at a **wavelength of 450 nm** and a reference wavelength between 620 nm and 650 nm **within 30 minutes of adding the stop solution.** Prior to measuring, slightly shake the microplate to ensure a homogeneous distribution of the solution.

* Please note that in case of deviating device specificiations customers are required to carry out their own validation.

Test performance using fully automated analysis devices

Sample dilution and test performance are carried out fully automatically using an analysis device. The incubation conditions programmed in the respective software authorised by EUROIMMUN may deviate slightly from the specifications given in the ELISA test instruction. However, these conditions were validated in respect of the combination of the DSX from Dynex and this EUROIMMUN ELISA. Validation documents are available on enquiry.
Automated test performance using other fully automated, open-system analysis devices is possible. However, the combination should be validated by the user.

Pipetting protocol

	1	2	3	4	5	6	7	8	9	10	11	12
A	**C 2**	**P 6**	**P 14**	**P 22**			**C 1**	**P 4**	**P 12**	**P 20**		
B	**pos.**	**P 7**	**P 15**	**P 23**			**C 2**	**P 5**	**P 13**	**P 21**		
C	**neg.**	**P 8**	**P 16**	**P 24**			**C 3**	**P 6**	**P 14**	**P 22**		
D	**P 1**	**P 9**	**P 17**				**pos.**	**P 7**	**P 15**	**P 23**		
E	**P 2**	**P 10**	**P 18**				**neg.**	**P 8**	**P 16**	**P 24**		
F	**P 3**	**P 11**	**P 19**				**P 1**	**P 9**	**P 17**			
G	**P 4**	**P 12**	**P 20**				**P 2**	**P 10**	**P 18**			
H	**P 5**	**P 13**	**P 21**				**P 3**	**P 11**	**P 19**			

The pipetting protocol for microtiter strips 1 to 4 is an example for the **semiquantitative analysis** of 24 patient samples (P 1 to P 24).
The pipetting protocol for microtiter strips 7 to 10 is an example for the **quantitative analysis** of 24 patient samples (P 1 to P 24).

The calibrators (C 1 to C 3), the positive (pos.) and negative (neg.) controls, and the patient samples have each been incubated in one well. The reliability of the ELISA test can be improved by duplicate determinations for each sample.
The wells can be broken off individually from the strips. This makes it possible to adjust the number of test substrates used to the number of samples to be examined and minimises reagent wastage.
Both positive and negative controls serve as internal controls for the reliability of the test procedure. They should be assayed with each test run.

Calculation of results

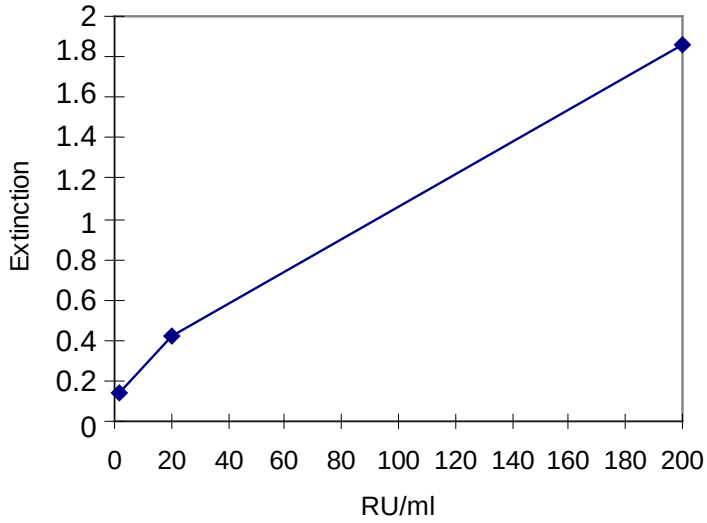
Semiquantitative: Results can be evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient sample over the extinction value of calibrator 2. Calculate the ratio according to the following formula:

$$\frac{\text{Extinction of the control or patient sample}}{\text{Extinction of calibrator 2}} = \text{Ratio}$$

EUROIMMUN recommends interpreting results as follows:

Ratio <1.0: negative
Ratio ≥1.0: positive

Quantitative: The calibration curve from which the concentration of antibodies in the patient samples can be taken is obtained by point-to-point plotting of the extinction values measured for the 3 calibrators against the corresponding units (linear/linear). Use “point-to-point” plotting for calculation of the calibration curve by computer. The following plot is an example of a typical calibration curve. Please do not use this curve for the determination of circulating immune complex concentrations in patient samples.



If the extinction for a patient sample lies above the value of calibrator 1 (200 RU/ml), the result should be reported as “>200 RU/ml”. It is recommended that the sample be re-tested at a dilution of 1:201. The result in RU/ml read from the calibration curve for this sample must then be multiplied by a factor of 4.

The upper limit of the normal range (cut-off value) recommended by EUROIMMUN is 20 relative units per millilitre (RU/ml). This corresponds to 4 µg Eq/ml (µg equivalents of heat-aggregated human IgG per ml). EUROIMMUN recommends interpreting results as follows:

<20 RU/ml:	**negative**
≥20 RU/ml:	**positive**

For duplicate determinations the mean of the two values should be taken. If the two values deviate substantially from one another, EUROIMMUN recommends retesting the samples.

For diagnosis, the clinical picture of the patient always needs to be taken into account along with the serological findings.

Test characteristics

Calibration: The calibration is performed in relative units (RU). Five RU/ml correspond to one µg Eq/ml (µg equivalents of heat-aggregated human IgG per ml).

For every group of tests performed, the extinction values of the calibrators and the relative units and/or ratio determined for the positive and negative controls must lie within the limits stated for the relevant test kit lot. A quality control certificate containing these reference values is included. If the values specified for the controls are not achieved, the test results may be inaccurate and the test should be repeated.

The binding activity of the antibodies and the activity of the enzyme used are temperature-dependent. It is therefore recommended using a thermostat in all three incubation steps. The higher the room temperature (+18°C to +25°C) during the incubation steps, the greater will be the extinction values. Corresponding variations apply also to the incubation times. However, the calibrators, controls and patient samples are subject to the same influences, with the result that such variations will be largely compensated in the calculation of the result.

Antigen: The microplate wells were coated with the complement factor C1q prepared from human serum. The circulating immune complexes bind to the C1q via the Fc fragment of the immunoglobulins. C1q is a glycoprotein composed of 18 polypeptide chains and consisting of three nonidentical subunits with M.W. of 29,000, 26,000 and 19,000 respectively. C1q is complexed with two C1r and two C1s molecules to form the first component of complement (C1). Activation of complement via the classical pathway is triggered by binding of C1q to immune complexes or to a variety of other activating substances. Subsequent to C1q binding, C1r and C1s are converted to proteolytic enzymes, which are responsible for continuation of activation via the classical pathway.

Linearity: The linearity of the CIC-C1q ELISA (IgG) was determined by assaying at least 4 serial dilutions of different patient samples. The CIC-C1q ELISA (IgG) is linear at least in the tested concentration range (8 RU/ml to 193 RU/ml).

Detection limit: The lower detection limit is defined as the mean value of an analyte-free sample plus three times the standard deviation and is the smallest detectable antibody titer. The lower detection limit of the CIC-C1q ELISA (IgG) is 0.1 RU/ml

Analytical specificity: The ELISA presented here specifically detects circulating immune complexes containing IgG with the ability to bind to human complement fragment C1q. Elevated concentrations of CIC-C1q are observed in up to 10% of blood donors without any clinical manifestations.

Interference: Haemolytic, lipaemic and icteric samples showed no influence on the result up to a concentration of 10 mg/ml for haemoglobin, 20 mg/ml for triglycerides and 0.4 mg/ml for bilirubin in this ELISA.

Reproducibility: The reproducibility of the test was investigated by determining the intra- and inter-assay coefficients of variation (CV) using 3 samples. The intra-assay CVs are based on 20 determinations and the inter-assay CVs on 4 determinations performed in 6 different test runs.

Intra-assay variation, n = 20		
Sample	**Mean value (RU/ml)**	**CV (%)**
1	10	7.3
2	17	8.0
3	115	4.8

Inter-assay variation, n = 4 x 6		
Sample	**Mean value (RU/ml)**	**CV (%)**
1	8	10.6
2	14	14.0
3	100	10.2

Sensitivity and specificity: The CIC-C1q levels were determined in 55 serum samples from patients with systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) and from apparently healthy blood donors (origin: Europe, China) using the EUROIMMUN ELISA and another commercially available ELISA (Bühlmann Laboratories) as reference. Of the analysed sera, the same 22 were positive and the same 30 were negative in both assays. Two sera were found positive by the EUROIMMUN ELISA only, and one serum by the reference ELISA (Bühlmann Laboratories) only. The sensitivity amounted to 96%, with a specificity of 94%.

Reference range: The CIC-C1q concentrations were determined in serum samples from a total of 250 apparently healthy blood donors (origin: Germany) using this EUROIMMUN ELISA. CIC-C1q was detected at concentrations ranging from 1 to 296 RU/ml. The 90.0 percentile of the reference interval was calculated to be 15 RU/ml and the 95.0 percentile was found to be 31 RU/ml.

Clinical significance

Immune complexes are protein aggregates that form via the binding of antibodies to antigens. They are continually formed as a result of the immune response. Since they are rapidly degraded, circulating immune complexes (synonym: circulating Ab-Ag complexes, CIC) are not normally detected in serum or only in low concentrations. If CIC are present in disease in such high concentrations that they cannot be quickly eliminated from the body, e.g. if phagocyte capacity is overwhelmed, accumulation of the immune complexes occurs. This can influence the coagulation system and lead to increased thromobocyte and erythrocyte aggregation. Through activation of the complement system CIC can induce inflammatory reactions and cause damage to organs, as is the case, for example, in glomerulonephritis, Crohn’s disease, ulcerative colitis, rheumatic diseases and viral or bacterial infections.

In the classical activation pathway the antigen-antibody complexes bind to the complement components C1, C4 and C2 and trigger the enzyme cascade. This is continued and finished with the components C3, C5, C6, C7, C8 and C9. The factors, which include C1q, act as indicators of immune complex diseases with complement use.

Circulating or extravascular immune complexes can become pathogenetically active. When immune complexes accumulate on or in tissues this can lead to inflammatory processes with corresponding clinical symptoms. The involvement of immune complexes can be detected in a range of immune complex-associated autoimmune diseases, such as systemic lupus erythematosus (CLE), rheumatoid arthritis (RA) and immune complex glomerulonephritis. Elevated CIC serum concentrations are also found in bacterial, viral and parasitic infections as post- and parainfectious immune complex diseases, as well as in allergies, chronic skin diseases and neurological diseases.

Although the detection of CIC is not specific for a particular disease these analyses can yield useful information about the immunopathology, course and prognosis of disease. The presence of CIC is an indicator of an overwhelmed immune defence or an autoimmune conflict, which should be clarified by targeted tests. In autoimmune, microbial or neoplastic diseases measurement of CIC is used as a marker for disease activity and also for assessing organ manifestation and for monitoring treatment. The test method of choice is ELISA, which allows quantitative in vitro determination of C1q-binding, IgG-containing CIC. Complement CIC are bound to immobilised purified C1q proteins. Persistently elevated CIC-C1q indicates chronic active disease. Normalisation is considered an indicator of successful treatment. It is often possible to correlate the CIC level with clinical symptoms and relapses. The WHO recommends always performing a second determination several weeks later in order to evaluate the persistence of the immune complexes in the circulation and thereby the pathological significance. The second determination also allows treatment to be monitored. With appropriate therapy a clear decrease in CIC should occur.

According to the WHO the following diseases are CIC-associated: rheumatic diseases (rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, mixed connective tissue disease, periarteritis nodosa, Felty's syndrome, Bechterew's disease, Reiter's disease), viral infections (hepatitis B, cytomegaly, mononucleosis, subacute and sclerosing panencephalitis), bacterial infections (infectious endocarditis, disseminated gonorrhoea, syphilis, streptococcus and meningococcus infections), parasitosis (malaria, schistosomiasis, trypanosomiasis, toxoplasmosis), glomerulonephritis, neoplastic diseases and other diseases such as Crohn's disease, ulcerative colitis, idiopathic interstitial pneumonia, cystic fibrosis, multiple sclerosis, thrombotic thrombocytopenic purpura, Behcet's syndrome Churg-Strauss syndrome and chronic liver diseases.

Literature references

1. Aziz M, Akhtar S, Malik A. **Evaluation of cell-mediated immunity and circulating immune complexes as prognostic indicators in cancer patients**. Cancer detection and prevention 22.2 (1997): 87-99.
2. Braun A, Sis J, Max R, Mueller K, Fiehn C, Zeier M, Andrassy K. **Anti-chromatin and anti-C1q antibodies in systemic lupus erythematosus compared to other systemic autoimmune diseases.** Scand J Rheumatol 36 (2007) 291-298.
3. EUROIMMUN AG. Stöcker W, Schlumberger W, Krüger C. **Alle Beiträge zum Thema Autoimmundiagnostik.** In: Gressner A, Arndt T (Hrsg.) Lexikon der Medizinischen Laboratoriumsdiagnostik. 2. Auflage. Springer Medizin Verlag, Heidelberg (2012).
4. Flierman R, Daha MR. **Pathogenic role of anti-C1q autoantibodies in the development of lupus nephritis--a hypothesis.** Mol Immunol 44 (2007) 133-138.
5. Oelzner P, Deliyska B, Fünfstück R, Hein G, Herrmann D, Stein G. **Anti-C1q antibodies and antiendothelial cell antibodies in systemic lupus erythematosus - relationship with disease activity and renal involvement.** Clin Rheumatol 22 (2003) 271-278.
6. O'Flynn J, Flierman R, van der Pol P, Rops A, Satchell SC, Mathieson PW, van Kooten C, van der Vlag J, Berden JH, Daha MR. **Nucleosomes and C1q bound to glomerular endothelial cells serve as targets for autoantibodies and determine complement activation.** Mol Immunol 49 (2011) 75-83.

7. Saadoun, David, et al. **Anti-C1q antibodies in hepatitis C virus infection.** Clinical & Experimental Immunology 145.2 (2006): 308-312.

8. Shoenfeld Y, Szyper-Kravitz M, Witte T, Doria A, Tsutsumi A, Tatsuya A, Dayer JM, Roux-Lombard P, Fontao L, Kallenberg CG, Bijl M, Matthias T, Fraser A, Zandman-Goddard G, Blank M, Gilburd B, Meroni PL. **Autoantibodies against protective molecules--C1q, C-reactive protein, serum amyloid P, mannose-binding lectin, and apolipoprotein A1: prevalence in systemic lupus erythematosus.** Ann N Y Acad Sci 1108 (2007) 227-239.

9. Smykał-Jankowiak K, Niemir ZI, Polcyn-Adamczak M. **Do circulating antibodies against C1q reflect the activity of lupus nephritis?** Pol Arch Med Wewn 121 (2011) 287-295.

10. Trouw LA, Groeneveld TW, Seelen MA, Duijs JM, Bajema IM, Prins FA, Kishore U, Salant DJ, Verbeek JS, van Kooten C, Daha MR. **Anti-C1q autoantibodies deposit in glomeruli but are only pathogenic in combination with glomerular C1q-containing immune complexes.** J Clin Invest 114 (2004) 679-688.

11. Walport MJ. **Complement and systemic lupus erythematosus.** Arthritis Res 4 (2002) 279-293.

12. Zabaleta-Lanz ME, Muñoz LE, Tapanes FJ, Vargas-Arenas RE, Daboin I, Barrios Y, Pinto JA, Bianco NE. **Further description of early clinically silent lupus nephritis.** Lupus 15 (2006) 845-851.