

INSTRUCTIONS FOR USE

Complement C4d

Semi-Quantitative test

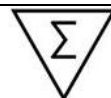
Enzyme immunoassay for assessment of C4d

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**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

REF

COMPL C4d RUO



PURPOSE OF RESEARCH PRODUCT

The Complement C4d assay is an enzyme-linked immunosorbent assay (ELISA) for the semi-quantitative determination of C4d in human plasma.

The product is intended for professional use. The result shall not be used for clinical diagnosis or patient management. FOR RESEARCH USE ONLY.

SUMMARY AND EXPLANATION

The complement system plays an essential role in autoimmune and infectious diseases. There are three pathways of complement activation; the classical, the alternative and the lectin pathway. The C4d protein is a product of the classical and the lectin pathways.

C4d, a degradation end product produced during complement C4 activation, had been recognized as a biomarker for its stability and strong association with antibody-mediated rejection in the 1990s and in the last twenty years, the potential importance of C4d in SLE as a tool for diagnosing and monitoring SLE was highlighted (1). Particularly, C4d associates with SLE nephritis (2). In primary Sjogren's syndrome (pSS) the level of C4d correlates with anti-SSB and κ/λ ratio and is suggested to be an appropriate marker of antibody response and complement activation in pSS patients with auto-antibodies (3,4). Plasma level of C4d has been shown to be significantly higher in patients with antibody-associated vasculitis with active disease compared with patients with lupus nephritis and normal controls (5). Peritubular C4d deposition is a significant predictor of long-term graft survival rates and to be of prognostic significance (6). C4d, is increased in biological samples from lung cancer patient and is associated with poor prognosis of lung cancer at a very early stage (7,8). C4d is increasingly recognized as a potential biomarker where antibodies can cause tissue damage, such as systemic autoimmune diseases. C4d has the potential to detect patients at risk for the consequences of antibody-mediated disease. Furthermore, the development of new therapeutics that block complement activation makes C4d a marker with potential to identify and monitor patients who may possibly benefit from these drugs (9). Complement assays based on detection of linear neopeptides has been reported to have an advantage compared to conformational epitopes, as it reduces the risk of false positives and increases specificity (10). This complement assay is based on the detection of the short linear C4d neopeptide exposed at the cleavage site of C4 after activation.

PRINCIPLE OF THE COMPLEMENT C4d ASSAY

The device is a colorimetric sandwich ELISA. Samples are diluted in assay diluent and 100 μ L diluted sample is transferred to the microtiter wells and incubated at room temperature for 60 minutes. During this first incubation C4d in the sample is captured by the anti-C4d-Neo monoclonal antibody, pre-coated on the surface of the microtiter wells. After washing to remove unbound material, a second horseradish peroxidase (HRP) labelled monoclonal antibody, that binds to both allelic variants of C4d (A and B), is added to the well. After incubation for 30 minutes the wells are washed again, and a substrate is added and incubated. The color development is stopped after 30 minutes, and the color is measured in a spectrophotometer. The color is directly proportional to the amount of C4d bound to the well. The amount of C4d is determined by comparison to the color development of the calibrator samples.

WARNINGS AND PRECAUTIONS

- For Research Use Only. Not for use in diagnostic procedures.
- Calibrators, controls, diluent, conjugate and wash solution contain ProClin 300 as a preservative. Never pipette by mouth or allow reagents or samples to come into contact with skin. Reagents containing ProClin may be irritating. Avoid contact with skin and eyes. In case of contact, flush with plenty of water. Handle ProClin 300 containing reagents as hazardous waste.
- The stop solution contains 0.5 M sulphuric acid. Do not allow the reagent to get into contact with the skin.
- The TMB substrate (3, 3', 5, 5'-tetramethylbenzidin) is toxic by inhalation, in contact with skin and if swallowed. Observe care when handling the substrate.
- Safety data sheet for all hazardous components contained in this kit is available on request from Euro Diagnostica.
- Plasma which are icteric, lipemic or hemolyzed may give erroneous results.

SPECIMEN COLLECTION

Blood samples are to be collected using aseptic venipuncture technique and EDTA plasma obtained using standard procedures. A minimum of 5 mL of whole blood is recommended. Centrifuge blood samples and transfer cell-free plasma to a clean tube. Plasma must be properly handled to prevent in vitro complement activation.

The centrifuged EDTA plasma may be kept at 4°C up to 8 hours and analysis should be performed within this timespan. For longer storage, plasma should be frozen at -70° C or lower. Samples should not be frozen and thawed more than twice.

KIT COMPONENTS AND STORAGE OF REAGENTS

- One frame with micro titer wells (12x8) coated with anti-C4d-Neo monoclonal antibody, sealed in a foil pack with a dessiccation bag.
- 1,5 mL Low control (LC). Ready to use
- 1,5 mL High control (HC). Ready to use
- 2x30 mL Diluent (Dil), red colour. Ready to use.
- 15 mL Conjugate containing HRP-labelled antibodies to C4d. Ready to use
- 15 mL Substrate TMB. Ready to use.
- 15 mL Stop solution (0.5M H₂SO₄). Ready to use
- 30 mL Wash solution, 30x concentrated
- 6 x 1,5 mL vials with calibrators containing human recombinant C4d,
- 0 ng/mL, 5 ng/mL, 25 ng/mL, 100 ng/mL, 200 ng/mL and 400 ng/mL. Ready to use

Please note:

All reagents in the kit are ready to use except the wash solution. The reagents should be stored at 2-8°C. Components from different lots shall not be mixed. When stored at 2-8°C the diluted wash solution is stable until the date of expiration of the kit.

Materials or equipment required but not provided

- Microplate reader with filter 450nm and 620nm
- Precision pipettes with disposable tips.
- Washer for strips, absorbent tissue, tubes and a timer.

PROCEDURE

1. Equilibrate reagents (microtiter plate, calibrators, controls, diluent, wash solution, conjugate, substrate and stop solution) to room temperature (20°C-25°C).
2. Dilute the wash solution 30x (ex. 30 ml concentrated wash solution + 870 ml dH₂O)
3. Thaw EDTA plasma samples at room temperature and put on ice.
4. Store thawed plasma samples on ice
5. Dilute samples 1/100 with diluent (ex. 10 µl plasma + 990 µl diluent)
Note: For good precision, avoid samples volumes less than 10 µl.
Note: For other dilutions see under heading Linearity/Recovery.
6. Transfer 100µL diluted sample, calibrator and controls to the microtiter wells in duplicates and incubate at room temperature for 60 minutes.
NOTE: transfer of samples, calibrators and controls to the plate should not take more than 15 minutes from the first to the last well to avoid drift of signal.
7. Wash 3 times with 300 µl diluted wash solution by filling and emptying the wells.
8. Add 100 µl conjugate to the wells. Incubate at room temperature for 30 minutes.
9. Wash 3 times with 300 µl diluted wash solution by filling and emptying the wells.
10. Add 100 µl substrate to the wells. Incubate at room temperature for 30 minutes.
11. Add 100 µl stop solution to the wells.
12. Read absorbance at 450 nm on the microplate reader. Read at 620 nm as reference wavelength.

CALCULATION OF RESULT

Subtract the 620 nm reference wavelength from the 450 nm wavelength and calculate mean absorbance values for all samples. Construct a calibrator curve by plotting the absorbance of the calibrators against the concentrations. Values of the six calibrators; 0 ng/ml, 5 ng/ml, 25 ng/ml, 100 ng/ml, 200 ng/ml and 400 ng/ml. A 4-parameters curve fit was used in the examples of this IFU (Figure 1). Users may select a different curve fit. Read the concentrations of the un-known samples against the calibrator curve.

NOTE: to calculate the concentration of C4d in the plasma sample, compensation for dilution must be done i.e. 100x according to the protocol above. If a different sample dilution is chosen, compensation for this should be done correspondingly.

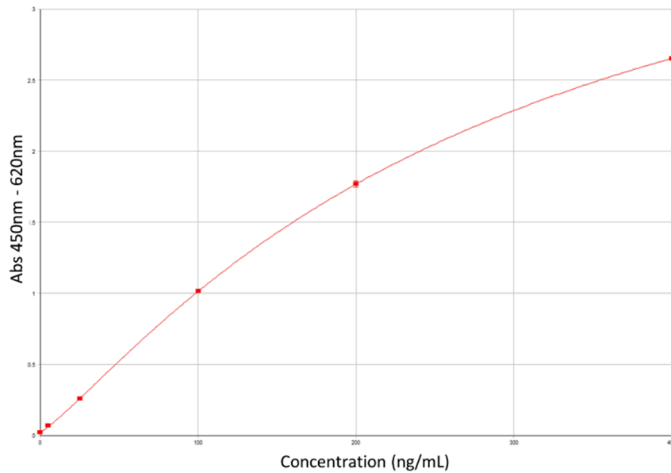


Figure 1. Example of a calibrator curve

Please Note: The figure above shows an example of a semi-quantitative standard curve and should not be used for actual subject sample interpretation.

QUALITY CONTROL

Absorbance for Calibrator 1 shall be <0.15

Absorbance for Calibrator 6 shall be >1.0

The high and low controls are intended to monitor for substantial reagent failure. If any of the controls are not within their respective range, the test should be considered as invalid and repeated. Limits are found on the certificate.

LIMITATIONS

This kit is for research use only and is not intended for diagnostic use. This kit has been used to test EDTA plasma. Other matrices have not been tested.

EXPECTED RESULTS

EDTA plasma from 140 apparently healthy blood donors were tested. Out of the 140 analyzed samples 92 had undetectable C4d concentration (<min), the remaining 48 samples had concentrations between 0.3ng/ml and 13.2 ng/ml. As not all samples had a numerical value, the 98% percentile was calculated manually and found to correspond to a concentration of 10 ng/ml. Note: After compensating for the 1/100 sample dilution the value corresponds to a neat sample titer of 1000ng/ml. A lower limit could not be calculated as a numerical value. Table 1.

It is recommended that each laboratory establish a reference range with samples commonly used since results may vary between different sample panels.

Table 1. Reference range of Complement C4d assay

Reference limits	n	Limit (ng/mL)
Lower limit	140	NA
Upper limit 98%	140	10

PRECISION

Between-run precision was determined by analyzing 9 EDTA plasma samples across the measuring range in 8 replicates at 4 occasions (Table 2). One of the test occasions was used for the calculations of within-run precision (Table 2).

Table 2. Within-run and between-run precision. Note: Results below are mean concentrations at 1/100 dilutions according to assay procedure.

Sample	Within-run ¹		Between-run ²	
	Mean conc. (ng/mL)	(%CV)	Mean conc. (ng/mL)	(%CV)
1	12	7	12	11
2	12	9	11	12
3	13	5	12	9
4	61	3	62	3
5	113	4	115	4
6	144	4	150	3
7	215	4	227	3
8	288	4	299	4
9	309	5	328	4

¹n=8 replicas ²n=4 runs

BATCH-TO-BATCH PRECISION

Batch-to-batch precision was determined by analyzing 12 EDTA plasma samples across the measuring range in three different kit batches (Table 3).

Table 3. Batch-to-batch precision. Note: Results below are mean concentrations at 1/100 dilutions according to assay procedure.

Sample	Batch 1 (ng/ml)	Batch 2 (ng/ml)	Batch 3 (ng/ml)	Mean (ng/ml)	SD	CV%
1	6	6	3	5	1,9	38*
2	6	7	4	5	1,7	34*
3	8	7	5	7	1,7	24*
4	55	45	41	47	7,3	16
5	111	85	85	94	15,1	16
6	106	99	94	100	5,9	6
7	109	107	95	104	7,7	7
8	147	144	132	141	8,1	6
9	190	127	160	159	31,3	20
10	208	193	188	197	10,1	5
11	284	184	225	231	50,5	22
12	248	228	236	238	10,3	4

* Plasma samples with absorbencies below 0.1

LINEARITY/RECOVERY

A dilution series was prepared for four EDTA plasma samples. A 1/100 dilution has been used in all reported results in the IFU but dilutions between 1/50 and 1/250 will yield accurate C4d concentrations (Table 4). Note that using a different sample dilution than 1/100 may shift the reference range. It is recommended that each laboratory establish a reference range with the dilution of choice.

Table 4. Linearity and recovery. Four C4d containing samples were diluted in diluent. Theoretical concentrations were calculated based on the 1/100 dilution.

Sample	Dilution factor	Mean measured conc.	Expected conc.	Recovery
1	50	237	255	93%
	100	128	128	100%
	200	69	64	108%
	250	59	51	116%
2	50	167	140	119%
	100	70	70	100%
	200	37	35	107%
	250	29	28	106%
3	50	106	130	82%
	100	65	65	100%
	200	37	32	115%
	250	29	26	113%
4	50	112	127	88%
	100	64	64	100%
	200	30	32	96%
	250	23	25	89%

LIMIT OF DETECTION

Limit of Detection (LOD) for the Complement C4d ELISA RUO has been estimated to 4,7 ng/mL, determined by dilution of C4d containing samples until signal to noise (diluent) was between 2 and 3.

CROSS REACTIVITY

Analytical specificity of the Complement C4d assay was challenged by analyzing complement factors genetically related to C4d. The following factors were analyzed: C4, C4b, C3, C3b, C3d, C5, SC5b-9 and C9. To challenge cross-reactivity the complement factors were tested at concentrations just above physiological concentrations (Figure 2).

Note that the Complement C4d assay detects both genetic variants of C4d (A and B).

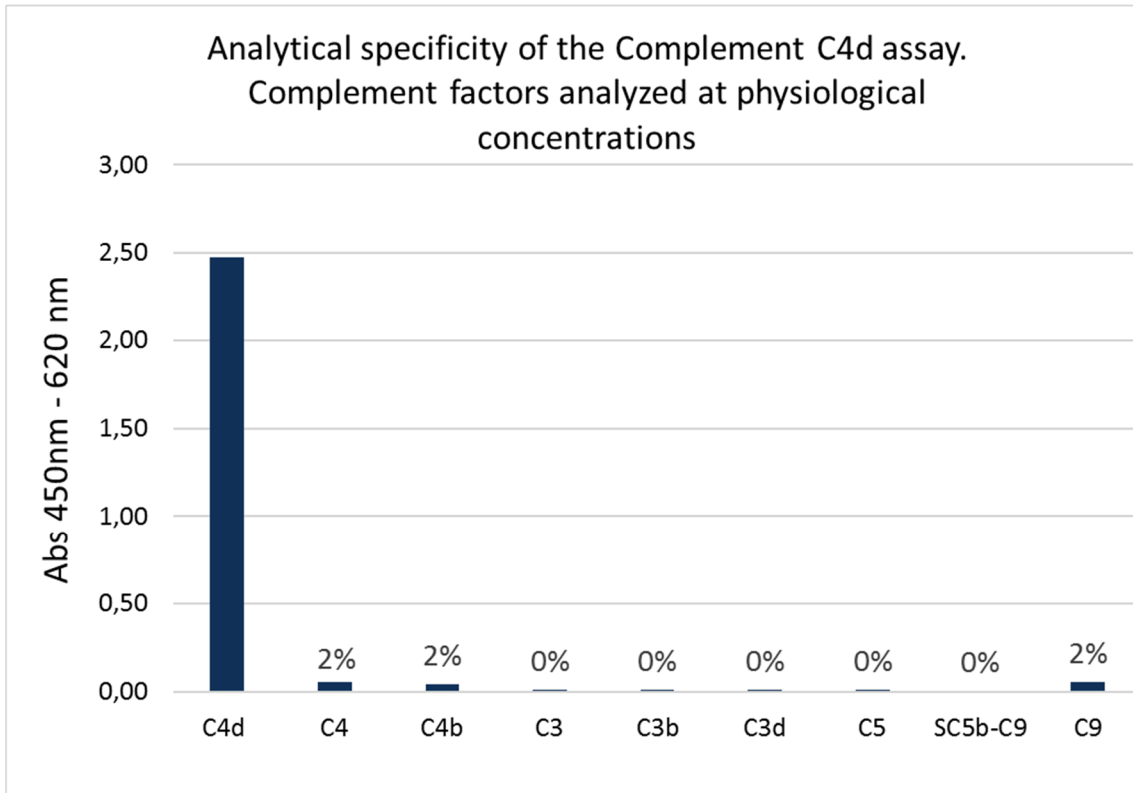


Figure 2. Cross reactivity with other complement factors in the C4d assay. Complement factors were tested at concentrations just above physiological concentrations.

HOOK EFFECT

No hook effect has been observed in Complement C4d ELISA up to 50 000ng/mL. Three normal EDTA plasma samples were spiked with purified C4d to final concentrations of 10 000ng/mL (High 1), 33 000 ng/mL (High 2) and 50 000ng/ml (High 3). The samples were diluted in 2-step dilution from 1/5 to 1/5120 in diluent.











REFERENCES:

1. **Juan Li, Zhuoli Zhang**, The Important Roles of C4d in Systemic Lupus Erythematosus, *Rheumatology (Sunnyvale)* 2014, 4: 135. 4:2
2. **Martin M, Smolağ KI, Björk A, Gullstrand B, Okrój M, Leffler J, Jönsen A, Bengtsson AA, Blom AM**, Plasma C4d as marker for lupus nephritis in systemic lupus erythematosus. *Arthritis Res Ther.* 2017 Dec 6;19(1):266. doi: 10.1186/s13075-017-1470-2.
3. **Sudzius, G., et.al**, 2014. Could the complement component C4 or its fragment C4d be a marker of the more severe conditions in patients with primary Sjogren's syndrome? *Rheumatol. Int.* 34, 235–241.
4. Okrój M, Johansson M, Saxne T, Blom AM. Analysis of complement biomarkers in systemic sclerosis indicates a distinct pattern in scleroderma renal crisis. Hesselstrand R. *Arthritis Res Ther.* 2016 Nov 18;18(1):267.
5. Gou, S.J., et.al., 2013. Circulating complement activation in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis. *Kidney Int.* 83, 129–137.
6. Herzenberg AM, Gill JS, Djurdjev O et al. C4d deposition in acute rejection: an independent long-term prognostic factor. *J Am Soc Nephrol*, 2002; 13: 234–241.
7. Ajona D, Pajares MJ, Corrales L, Perez-Gracia JL, Agorreta J, Lozano MD, et al. (2013) Investigation of Complement Activation Product C4d as a Diagnostic and Prognostic Biomarker for Lung Cancer. *J Natl Cancer Inst* 105(18):1385–93.
8. Ajona D, Okrój M, Pajares MJ, Agorreta J, Lozano MD, Zulueta JJ, Verri C, Roz L, Sozzi G, Pastorino U, Massion PP, Montuenga LM, Blom A. Complement C4d-specific antibodies for the diagnosis of lung cancer., *Plo R. Oncotarget.* 2017 Dec 26;9(5):6346-6355. doi: 10.18632/oncotarget.23690. eCollection 2018 Jan 19.
9. Cohen D, et.al.. Pros and cons for C4d as a biomarker. *Kidney Int.* 2012 Apr;81(7):628-39.
10. Blom AM, Österborg A, Mollnes TE, Okroj M. Antibodies reactive to cleaved sites in complement proteins enable highly specific measurement of soluble markers of complement activation. *Mol Immunol.* 2015 Aug;66(2):164-70. doi: 10.1016/j.molimm.2015.02.029. Epub 2015 Mar 18.








TROUBLESHOOTING

Problem	Possible causes	Solution
Calibrator or control values out of range	Incorrect temperature, timing or pipetting, reagents are not mixed	Check that the time and temperature were correct. Repeat test.
	Cross contamination of controls	Pipette carefully.
	Optical pathway is not clean.	Check for the dirt or air-bubbles in the wells. Wipe plate and reread.
All test results negative	One or more reagents are not added or added in wrong sequence.	Recheck procedure. Check for unused reagents. Repeat test.
	Antigen coated plate is inactive	Check for obvious moisture in unused wells. Wipe plate bottom and reread.
All test results yellow.	Contaminated buffers or reagents.	Check all solutions for turbidity.
	Washing solution is contaminated.	Use clean container. Check the quality of water used for preparation of solution.
	Improper dilution of plasma.	Repeat test.
Poor precision.	Pipette delivery CV >5% or samples not mixed.	Check the calibration of pipette. Use reproducible technique. Avoid air bubbles in pipette tip.
	Plasma or reagents are not mixed sufficiently or not equilibrated to room temperature.	Mix all reagents gently but thoroughly and equilibrate to room temperature.
	Reagent addition is taking too long time, inconsistency in timing intervals.	Develop consistent uniform technique and use multi-tip device or auto-dispenser to decrease time.
	Optical pathway not clean.	Check for air bubbles in the wells. Wipe plate bottom and reread.
	Washing not consistent, trapped bubbles, washing solution left in the wells.	Check that all wells are filled and aspirated uniformly. Dispense liquid above level of reagent in the well. After last wash, empty the wells by tapping the strip on an absorbent tissue.
	The complement system has self-activated in the sample	Draw new sample and keep strict to the timelines and temperatures recommended in specimen collection section

EXPLANATION OF SYMBOLS

	<p>Batch number.</p>
	<p>Catalogue number.</p>
	<p>Use-by date.</p>
	<p>Temperature limit.</p>
	<p>Biological risk.</p>
	<p>Consult instructions for use.</p>
	<p>Warning.</p>
	<p>Manufacturer.</p>
	<p>Content sufficient for 96 tests.</p>
	<p>Corrosive.</p>

EXPLANATION OF SYMBOLS

Ab	Antibody.
DIL	Diluent. 
CONJ	Conjugate 
BUF WASH 30X	Wash solution 30x conc. 
SUB TMB	Substrate TMB
CONTROL L	Low control 
CONTROL H	Highcontrol 
CAL 1-6	Calibrator 
H₂SO₄ 0.5M	Stop solution 



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