Complement C4d Flexi

A Gyrolab® system application

For research and professional use only. Not for use in diagnostic procedures.

This application note contains a suggested protocol and performance data.
Each individual laboratory must set up their own method and perform relevant validations.

Background

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 as a tool for diagnosing and monitoring SLE was highlighted (1). Particularly, C4d associates with SLE nephritis (2). In primary Sjögren'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. 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 neoepitopes have been reported to have an advantage compared to conformational epitopes, as it reduces the risk of false positives and increases specificity (10).

Purpose

The purpose of the Complement C4d assay and the Complement C4d Flexi reagents is to detect the short linear C4d neoepitope, exposed at the cleavage site of C4 after activation, in EDTA plasma samples. The Complement C4d Flexi application note allows users to independently set up, customize and optimize a Complement C4d Flexi assay based on Svar Life Science's Complement C4d assay on the Gyrolab® automated platforms (Gyros Protein Technologies) or another platform of their choice using Svar Life Science's Flexi products.

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

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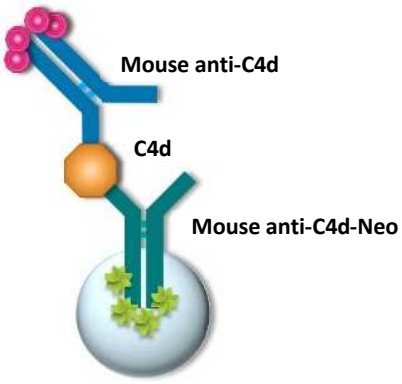
Assay design

It is recommended to set up Complement C4d Flexi assays as 3-step sandwich assays with the capture and detector antibodies used in their designated functions to ensure optimal specificity for the C4d neoepitope in EDTA plasma samples. The recommended assay design is also used in Svar Life Science's Complement C4d ELISA (COMPL C4D RUO). A recommended Gyrolab® assay protocol is described below, allowing for automated C4d quantification using Svar Life Science's proprietary C4d antibodies.



Materials and methods

The following semi-quantitative Complement C4d Flexi assay was developed on a Gyrolab® xPlore using the Flexi reagents listed in Table 1 combined with additional materials and equipment listed in Table 2. The assay has not been validated on a multi-disc instrument. This protocol may not be directly transferrable to other platforms without further optimization and validation.

Table 1. Flexi reagents required.

Reagent	Volume	Concentration	Product code
Complement C4d Flexi Capture antibody (CAP Ab)	16 µL	1.25 mg/mL	FX1342
Complement C4d Flexi Detection antibody (DET Ab)	16 µL	1.25 mg/mL	FX1343
Complement Flexi Diluent (DIL)	32 mL	-	FX1344
Complement C4d Flexi Calibrator stock (CAL)	70 µL	400 ng/mL	FX1345
Complement C4d Flexi Low control (CONTROL L)	35 µL	CoA	FX1346
Complement C4d Flexi High control (CONTROL H)	35 µL	CoA	FX1347

Store all the Flexi reagents at 2–8°C. Once conjugated, follow the antibody conjugate storage instructions from the supplier of the respective conjugation kits. Reagents of different lots or different conjugation reactions shall not be mixed. Dispose of the reagents as hazardous waste.

Table 2. Materials or equipment required but not provided by Svar Life Science.

Item	Supplier	Product code
Gyrolab® platform (e.g. xPlore)	Gyros Protein Technologies	-
Biotinylation kit	See Gyrolab® User Guide	See Gyrolab® User Guide
Alexa Fluor® 647-labelling kit	See Gyrolab® User Guide	See Gyrolab® User Guide
Bioaffy™ 1000 CD	Gyros Protein Technologies	P0004253
PBS-T	See Gyrolab® User Guide	See Gyrolab® User Guide
Rexxip® F	Gyros Protein Technologies	P0004825
Gyrolab® wash buffer pH 11	Gyros Protein Technologies	P0020096
PCR Plate 96	Gyros Protein Technologies	P0004861
Microplate foil	Gyros Protein Technologies	P0003313
Microplate foil adapter	Gyros Protein Technologies	P0003697
Precision pipettes with disposable tips	-	-

Complement C4d Flexi Gyrolab® protocol

Before starting a Complement C4d Flexi Gyrolab® assay, equilibrate all materials to room temperature and perform the assay at room temperature. The assay protocol is summarized in Table 3.

Table 3. Complement C4d Flexi Gyrolab® protocol summary.

Capture	100 µg/mL biotinylated* capture antibody in PBS-T
Detection	10 µg/mL Alexa Fluor® 647-labeled* detection antibody in Rexxip® F
Analyte	C4d in EDTA plasma
Standard curve	Calibrator stock in diluent: 400, 200, 100, 25, 5, 0 ng/mL
CD-type	Bioaffy™ 1000 CD
Method	1000-3W-006-A
Wash buffer 1	PBS-T
Wash buffer 2	Gyrolab® wash buffer pH 11
PMT-setting	PMT 1%
Standard fit model	5-parameter logistic (5PL) curve fit excluding the blank
Expected dynamic range	5–400 ng/mL
Sample dilution	1:25 (MRD) to 1:50 in diluent. Up to 1:128 can be used if required.

*Conjugation of antibodies is required before use, see Gyrolab® User Guide.

Antibody conjugation

The capture antibody, an anti-C4d-Neo monoclonal antibody, should be biotinylated according to the Gyrolab® standard protocols (Gyrolab® User Guide) prior to use in Gyrolab® assays. Centrifuge the antibody solution before conjugation to ensure sufficient antibody recovery and mix carefully with a pipette, avoid formation of foam, before aspirating the solution. In the Gyrolab® assay, the conjugated capture antibody is diluted to 100 µg/mL with PBS-T.

The detection antibody, an anti-C4d antibody, should be labeled with Alexa Fluor® 647 according to the Gyrolab® User Guide prior to use in Gyrolab® assays. Centrifuge the antibody solution before conjugation to ensure sufficient antibody recovery and mix carefully with a pipette, avoid formation of foam, before aspirating the solution. In the Gyrolab® assay, the conjugated capture antibody is diluted to 10 µg/mL with Rexxip® F.

Sample preparation

EDTA plasma samples should be equilibrated to room temperature and freshly diluted just before running the assay as the samples may have residual complement activity. Undiluted samples can be kept at 2–8°C for up to 8 h. All samples should be centrifuged prior to dilution (Gyrolab® User Guide). See **Specimen collection** for detailed information on collecting and storing specimen.

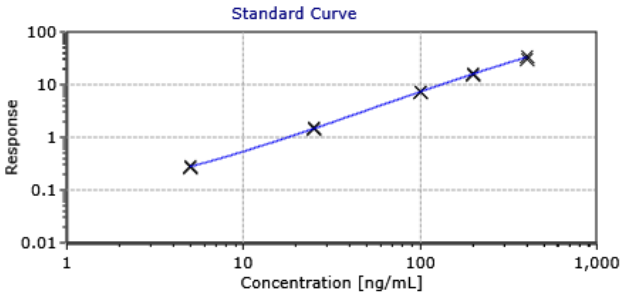
The centrifuged samples should be diluted with the Complement Flexi diluent to a minimum recommended dilution (MRD) of 1:25 up to 1:50. If required, depending on specimen analyte concentration, individual samples can be diluted further up to 1:128, but 1:25 to 1:50 dilution should suffice for most specimen. It is recommended, when possible, to use the same dilution factor within a given study to reduce variation. When diluting samples, it is recommended to dilute at least 10 µL of sample (e.g. 10 µL sample + 240 µL diluent for 1:25 dilution). To calculate the concentration of C4d in the plasma sample, the measured analyte concentration should be compensated for the dilution factor, e.g. 25x if using the MRD above. It is recommended to measure all samples, calibrators and controls in duplicates to ensure that results are precise.

Standard curve

Recombinant human C4d is used as the assay standard. The standard curve should be diluted in a dilution series from the calibrator stock (400 ng/mL) using the Complement Flexi diluent, see recommended standard curve in Table 4. Analyte concentrations are interpolated from the recommended standard curve using the PMT 1% response and a 5-parameter logistic (5PL) curve fit excluding the blank. It is recommended to include the high and low control samples in each assay run to verify the performance of the assay. Using the recommended assay protocol, the controls should measure within the limits given on their respective certificate of analysis.

Table 4. Recommended standard curve preparation. Note: the graph shows an example of a semi-quantitative standard curve and should not be used for actual subject sample interpretation.

Calibrator	Conc. (ng/mL)	Calibrator solution	Diluent
A	400	10 µL	-
B	200	10 µL A	10 µL
C	100	10 µL B	10 µL
D	25	10 µL C	30 µL
E	5	10 µL D	40 µL
F	Blank	-	10 µL



Specimen collection

It is recommended to measure C4d levels in EDTA plasma. Blood samples are to be collected using aseptic venipuncture technique and EDTA plasma is 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.

Matrices other than EDTA plasma have not been tested, and the user should carefully validate the assay's performance in other matrices and pay special attention to matrix effects and false positives derived from potential in vitro complement activation.

Assay performance

All results reported below were generated using the recommended Complement C4d Flexi Gyrolab® assay protocol as described above.

Working range

Table 5. Assay working range. The lowest (LLOQ) and highest (ULOQ) concentrations of the standard curve that gave a Total Error (% absolute bias + % CV) < 30 % in three repeated assessments were assigned as LLOQ and ULOQ.

LOD (ng/mL)	LLOQ (ng/mL)	ULOQ (ng/mL)
5	5	400

Precision

Between-run precision was determined by analyzing 10 C4d-containing EDTA-plasma samples, some of which were spiked with calibrator material, at 1:25 dilution across the measuring range in 4 replicates at 3 occasions (Table 6). One of the test occasions was used to calculate within-run precision (Table 6).

Table 6. Within-run and between-run precision. Note: Results below are mean concentrations at 1:25 sample dilution. [1]n=4 replicates, [2]n=3 runs

Sample	Within-run[1]		Between-run[2]	
	Mean conc. (ng/mL)	CV (%)	Mean conc. (ng/mL)	CV (%)
1	11.4	7.6	10.3	15.8
2	12.6	9.0	10.8	15.7
3	30.7	10.6	23.8	27.2
4	40.0	5.3	33.7	17.5
5	52.8	1.6	53.8	2.1
6	61.7	3.0	62.7	8.8
7	89.8	2.4	91.0	3.1
8	96.2	2.8	97.7	1.3
9	98.1	1.9	98.3	5.6
10	186.3	3.3	185.1	2.6

Linearity

A dilution series was prepared for 4 C4d-containing EDTA-plasma and 1 zymosan-activated serum sample, the latter containing very high levels of native C4d. As demonstrated here, dilutions between approx. 1:25 and 1:128 will yield accurate C4d results (Table 7).

Table 7. Linearity and recovery. Five C4d-containing samples were diluted in diluent to a wide range of dilutions. A set of relevant dilutions are reported in the below table. Dilutions outside this range may not be linear. Theoretical true concentrations were calculated based on the 1:25 dilution.

Sample	Dilution factor	Mean measured conc. (ng/mL)	Expected conc. (ng/mL)	Recovery (%)
1	25	18.9	18.9	100.0
	57	7.9	8.3	95.2
	128	N/A	N/A	N/A
2	25	58.1	58.1	100.0
	57	25.6	25.5	100.4
	128	10.5	11.3	92.9
3	25	23.8	23.8	100.0
	57	10.4	10.4	100.0
	128	N/A	N/A	N/A
4	25	55.8	55.8	100.0
	57	24.4	24.5	99.6
	128	10.1	10.9	92.7
5	25	126.3	126.3	100.0
	57	61.4	55.4	110.8
	128	30.6	24.7	123.9

Note: The use of different dilution factors may be required depending on assay setup, sample type as well as sample analyte concentration. Samples measuring above the ULOQ should be diluted further.

Cross-reactivity

The antibody pair used in this protocol detects both genetic variants of C4d (A and B, data not shown) but is highly specific for C4d.

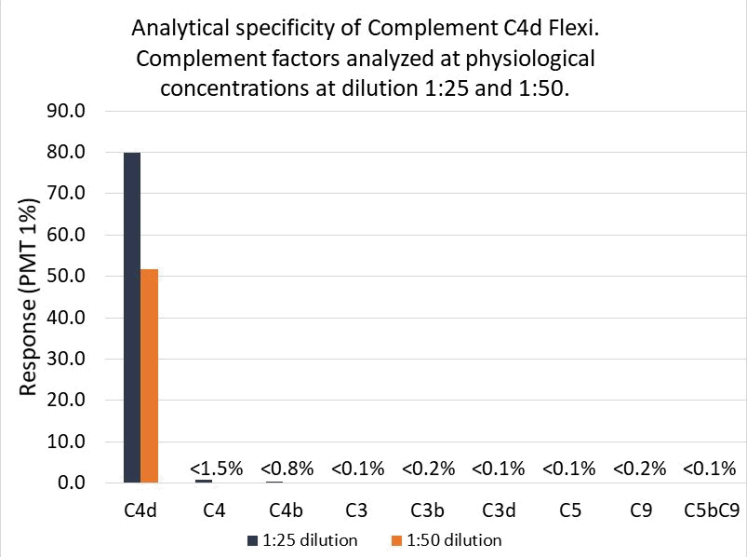


Figure 1. Cross-reactivity with other complement factors in the Complement C4d Flexi Gyrolab® assay. Complement factors were tested at 1:25 and 1:50 dilution relative to physiological concentrations. Percentage comparisons are between C4d at 1:50 dilution and other complement factors at 1:25 dilution.

Hook effect

No hook effect has been observed in the assay when using up to 40 000 ng/mL recombinant C4d diluted in diluent.

Recommendations

It is recommended that users determine the concentrations of antibodies after conjugation and titrate their signal against the standard curve and controls to ensure that the assay is functional.

Usage on other platforms or with altered protocols should be carefully validated by the user.

If customizing this assay, it is important to screen matrices and assess backgrounds, in particular for disease-specific matrices. Assay parameters should be validated in-house. Data given in this document should only be considered as a guidance.

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