Calprotectin 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

Various types of organic diseases in the gastrointestinal tract may cause damage to the intestinal epithelial lining (mucosa layer). Such damage may vary from increased permeability of the mucosa to inflammation and ulcerations. The bowel content is rich in bacteria and other microorganisms releasing substances which may be toxic or chemotactic, i.e. they stimulate leukocytes, in particular polymorphonuclear neutrophilic granulocytes (PMN), to migrate into the gut lumen where they release their contents including antimicrobial substances like Calprotectin. This protein constitutes about 60% of total proteins in the cytoplasm of PMNs (1) and can reliably be estimated in fecal samples stored for up to seven days at ambient temperature (2). Calprotectin is a 36 kilodalton calcium and zinc-binding protein (3), produced by PMNs, monocytes and squamous epithelial cells except those in normal skin (4, 5). After binding of calcium, it can resist degradation by leukocytic and microbial enzymes (2, 6). By competing with different enzymes for limited, local amounts of zinc, Calprotectin can inhibit many zinc dependent enzymes (7) and thereby kill microorganisms or animal and human cells in culture (8, 9). Different types of disease, for instance bacterial infections, rheumatoid arthritis or cancer lead to activation of PMNs and increased levels of Calprotectin in plasma, cerebrospinal fluid, synovial fluid, urine or other human materials (10). It is of special importance that the concentration of Calprotectin in faeces is correlated with the number of PMNs migrating into the gut lumen (11), and that it can be detected reliably even in small (less than one gram) random stool samples (2, 12). Furthermore, organic diseases of the bowel give a strong Calprotectin signal, i.e. elevations are regularly five to several thousand times the upper reference in healthy individuals (2, 13, 14, 15), indicating intestinal inflammation.

The concentration of Calprotectin measured in stools is a non-invasive and objective marker; it can be used to determine disease activity and response to treatment of IBD, and to tell when a true remission has been achieved. Calprotectin can also be analyzed in serum and plasma samples, and high Calprotectin levels have been found in patients with bacterial infections, sepsis and inflammatory conditions like rheumatoid arthritis (RA) and Systemic Lupus Erythematosus (SLE).

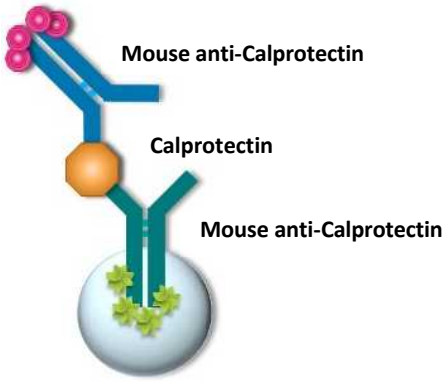
Purpose

The purpose of this Calprotectin assay and the Calprotectin Flexi reagents is to detect Calprotectin in fecal extracts and/or EDTA plasma samples. This application note and the Calprotectin Flexi reagents comprise the essential requirements to independently set up, customize and optimize the Calprotectin assay on the Gyrolab® automated platform (Gyros Protein Technologies) or another platform of your choice.

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

Assay design

It is recommended to set up Calprotectin Flexi assays as 3-step sandwich assays with the included antibodies in their indicated functions as capture and detector to ensure optimal specificity for detection of native Calprotectin in stool extract or plasma samples. The recommended assay design has also been used in other diagnostic tests developed by Calpro AS (a member of the Svar Life Science group of companies). A recommended Gyrolab® assay protocol is described below, allowing for automated Calprotectin quantification using Svar Life Science's proprietary antibodies.



Materials and methods

The following semi-quantitative Calprotectin assay was developed on a Gyrolab® xPlore using the Calprotectin 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.

Reagents	Volume	Concentration	Product code
Calprotectin Flexi Capture antibody (CAP Ab)	16 µL	1.25 mg/mL	FX1348
Calprotectin Flexi Detection antibody (DET Ab)	16 µL	1.25 mg/mL	FX1349
Calprotectin Flexi Diluent (DIL)	3x30 mL	-	FX1353
Calprotectin Flexi Calibrator stock (CAL)	50 µL	1500 ng/mL	FX1350
Calprotectin Flexi Low control (CONTROL L)	20 µL	CoA	FX1352
Calprotectin Flexi High control (CONTROL H)	20 µL	CoA	FX1351

Store all 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 No.
Calpro EasyExtract™	Calpro AS	CALP0170
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™ 200 CD	Gyros Protein Technologies	P0004180
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	-	-

Calprotectin Flexi Gyrolab® protocol

Before starting a Calprotectin 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. Calprotectin 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	Calprotectin in fecal extract (prepared using Calpro EasyExtract™)
Standard curve	Calibrator stock in diluent: 1500, 500, 125, 62.5, 31.3, 7.8, 1.95, 0 ng/mL
CD-type	Bioaffy™ 200 CD
Method	200-3W-002-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	1.95–1500 ng/mL
Sample dilution	1:20 (MRD) to 1:100 in diluent. Up to 1:1139 can be used if required.

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

Antibody conjugation

The capture antibody, an anti-Calprotectin 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 in PBS-T.

The detection antibody, an anti-Calprotectin monoclonal 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

Fecal extracts and EDTA plasma samples should be equilibrated to room temperature and freshly diluted just before running the assay. Undiluted samples can be kept at 2–8°C for up to 7 days. All samples should be centrifuged prior to dilution (Gyrolab® User Guide). See **Specimen collection** for detailed information on collecting and storing specimen.

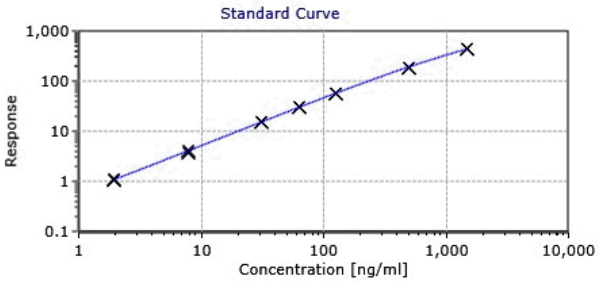
Dilute the centrifuged samples using Calprotectin Flexi diluent with a recommended dilution (MRD) of 1:100 for fecal extracts or 1:20 for plasma samples. If required, depending on specimen analyte concentration, individual samples can be diluted further up to 1:1139, but 1:20 to 1:100 dilution should be adequate for most specimen. It is recommended, when possible, to use the same dilution factor within a given study to reduce variation. Dilution of other sample specimen is to be evaluated by the user. To calculate the concentration of Calprotectin in fecal extracts prepared with Calpro EasyExtract™, the measured analyte concentration should be multiplied by factor 5 for the Calprotectin concentration in mg/kg, other samples should be multiplied by their according dilution factor. Other matrices than fecal extract and EDTA plasma have not been tested in this application note and should be evaluated carefully. It is recommended to measure all samples, calibrators and controls in duplicates to ensure that results are precise.

Standard curve

Native purified human Calprotectin is used as the assay standard. The standard curve should be diluted in a dilution series from the calibrator stock (1500 ng/mL) using Calprotectin Flexi diluent, see recommended standard curve in Table 4. Analyte concentrations are interpolated 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 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 their respective limits given on the 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	1500	10 µL	-
B	500	5.0 µL A	10 µL
C	125	5.0 µL B	15 µL
D	62.5	10 µL C	10 µL
E	31.3	10 µL D	10 µL
F	7.81	5.0 µL E	15 µL
G	1.95	5.0 µL F	15 µL
H	Blank	-	5.0 µL



Specimen collection

All samples analyzed in this application note were either fecal extracts or EDTA plasma samples. 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. Fecal extracts from stool samples were prepared using the Calpro EasyExtract™ device from Calpro AS, Norway (CALP0170). All samples may be kept at 2-8°C up to 7 days and analysis should be performed within this timespan. For longer storage, fecal extract and plasma should be frozen at -70° C or lower. Samples should not be frozen and thawed more than twice.

Matrices other than fecal extracts and EDTA plasma have not been tested, and the user should carefully validate the assay's performance in other matrices.

Assay performance

All results reported below were generated using the recommended Calprotectin 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)
2	2	1500

Precision

Between-run precision was determined by analyzing 7 samples (4 fecal extracts 1:100 diluted, 2 fecal extracts spiked with native purified Calprotectin and 1 plasma pool 1:20 diluted). All samples were analyzed in 3 replicates at 3 different occasions within 1 week (Table 6). At one of the test occasions samples were analyzed in 8 replicates and used for the calculations of within-run precision (Table 6).

Table 6. Within-run and between-run precision. Note: Results below are mean concentrations at 1:100 sample dilution for fecal extracts and 1:20 dilution of plasma pool. ([1]n=8 in 3 runs, [2]n=3)

Sample	Within-run[1]		Between-run[2]	
	Mean conc. (ng/mL)	CV (%)	Mean conc. (ng/mL)	CV (%)
1	3.03	15	2.79	19
2	67.1	5.2	63.7	5.0
3	6.77	2.7	6.73	2.2
4	369	3.0	356	1.3
5	13.8	5.4	13.5	4.5
6	53.0	2.9	53.1	3.6
7	103	1.6	104	6.2

Linearity

A dilution series was prepared from 3 fecal extracts and 1 fecal extract spiked with analyte, the latter containing high levels of native Calprotectin. As demonstrated here, dilutions around 1:100 will yield accurate Calprotectin results and should suffice for most specimen (Table 7).

Table 7. Linearity and recovery. Four Calprotectin-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 lowest dilution with concentration below the ULOQ.

Sample	Dilution factor	Expected conc. (ng/mL)	Measured conc. (ng/mL)	Recovery (%)
1	171	67.4	71.2	106
	114	101	101	100
	51	228	263	116
	23	512	512	100
2	171	2.79	2.38	85
	76	6.28	4.46	71
	34	14.1	12.8	91
	15	31.8	31.8	100
3	1139	36.7	38.7	105
	506	82.7	86.3	104
	225	186	202	109
	100	419	419	100
4	128	35.5	35.5	100
	64	71.0	65.5	92
	16	284	255	90
	8	568	568	100

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.

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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