RIDASCREEN® Calprotectin

Art. No.: G09036

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1. Intended use

For *in vitro* diagnostic use. RIDASCREEN® Calprotectin is an enzyme immunoassay for the quantitative determination of human calprotectin in stool samples.

2. Summary and explanation of the test

Calprotectin, a calcium-binding protein produced in cells such as neutrophils, is released during inflammation.
Fecal calprotectin serves as a biomarker of gastrointestinal inflammation and neoplasia.

Calprotectin (also known as MRP-8/14, calgranulin A/B and S100A8/A9) is a calcium and zinc-binding protein of the S100 protein family.
Calprotectin binds calcium under physiological conditions. Because of its calcium binding properties, calprotectin is a stable protein that is highly resistant to heat and proteolysis.
Calprotectin is produced by both neutrophils (neutrophil granulocytes) and monocytes. In neutrophils, the calprotectin concentration in the cytosol ranges from 5 to 15 mg/l, which accounts for about 60 % of the soluble cytosolic protein fraction and 5 % of the total protein fraction of these cells. The biological function of calprotectin is not completely understood. It is assumed that calprotectin serves a biological function in protecting cells from leukocytic and bacterial proteases. Calprotectin is also presumed to have antibacterial properties by virtue of its zinc-binding capacity. In addition, calprotectin exhibits both intra- and extracellular regulatory functions in inflammatory processes.

In the presence of an inflammatory bowel disease, neutrophils influx into the intestinal lumen and release calprotectin, which is excreted in the feces. The concentration of calprotectin in the feces correlates with the number of neutrophils that migrated to and released calprotectin in the intestinal lumen. Consequently, the fecal calprotectin concentration can be used as a measure of the number of neutrophils in the bowel lumen and as an indicator of the severity of intestinal inflammation.

Clinically, the special relevance of fecal calprotectin measurement lies in its capacity for reliable diagnosis of inflammatory bowel disease. In many cases, chronic inflammatory bowel disease (IBD) produces symptoms that are very difficult to distinguish from those of irritable bowel syndrome (IBS). Fecal calprotectin measurement provides a reliable indication of the presence of inflammatory bowel disease. Through the use of this biomarker, many patients with irritable bowel syndrome can therefore be spared unnecessary colonoscopy.
In a number of publications, fecal calprotectin concentrations have been shown to be strongly correlated with histological and endoscopic parameters of disease activity in patients with chronic inflammatory bowel disease. Therefore, measurement of fecal calprotectin provides a

means of objectively assessing the response to treatment of chronic inflammatory bowel disease and for monitoring these patients during clinical remission to enable the early detection and treatment of relapses of IBD.

Fecal calprotectin should be evaluated:

- For reliable differentiation between chronic inflammatory bowel disease and irritable bowel syndrome
- For early detection of flares of inflammatory bowel disease
- To objectively document the severity of inflammation for response monitoring during the course of treatment
- As a marker of acute intestinal inflammation

3. Test principle

In RIDASCREEN® Calprotectin specific antibodies are used in a sandwich-type method. Monoclonal antibodies against human calprotectin epitopes are applied to the surface of the well in the microwell plate. A suspension of the stool sample which is to be tested is pipetted into the well of the microwell plate and incubated. After a washing step follows a second incubation phase together with a monoclonal antibody which is conjugated with horseradish peroxidase. In the presence of calprotectin, a sandwich complex forms which is made up of immobilised antibodies, calprotectin and conjugated antibodies. Unattached enzyme-labelled antibodies are removed during a further washing phase. After adding the substrate, the attached enzyme changes the colour of the previously colourless solution in the wells of the microwell plate to blue if the test is positive. On adding the stop reagent, the colour changes from blue to yellow. The absorbance is proportional to the concentration of calprotectin present in the sample.

4. Reagents provided

One kit is sufficient for 96 determinations.

Plate	96 det.	Microwell plate, 12 microwell strips (can be divided) in the strip holder; coated with monoclonal antibodies (mouse) against human calprotectin
Extract	2x 100 ml	Extraction buffer (3-fold conc.); Tris buffer; contains 0.05% NaN_3
Diluent | 3	100 ml	Sample dilution buffer, protein-buffered NaCl-solution; contains 0.1% NaN_3; ready for use; coloured red
Wash	100 ml	Wash buffer (10-fold conc.) , phosphate-buffered NaCl solution; contains 0.1 % Thimerosal and 0.1% detergent
Calibrator *transparent lid*	1 ml	Calibrator (1-point calibration procedure); contains 0.1% ml NaN_3; ready for use
Standard 1 to Standard 5 *white lid*	1 ml	5 Standards (standard curve procedure); Calprotectin-concentrations of the standards 1 to 5: 19.5 / 56 / 95 / 275 / 800 mg/kg; contains 0,1% NaN_3; ready for use
Control | + *red lid*	1 ml	Positive control; contains 0.1% ml NaN_3 ; ready for use
Low control | + *green lid*	1 ml	Low positive control; contains 0.1% ml NaN_3 ; ready for use
Conjugate	12 ml	Peroxidase conjugated, monoclonal antibody (mouse) in stabilised protein solution; ready for use
SeroSC	12 ml	Substrate; Hydrogen peroxide / tetramethylbenzidine (TMB); ready for use
Stop	12 ml	Stop reagent, 1 N sulphuric acid; ready for use

5. Storage instructions

All reagents must be stored at 2 - 8 °C and can be used until the expiry date printed on the label. The diluted wash buffer can be used for a maximum of 4 weeks when stored at 2 - 8 °C. The diluted extraction buffer can be used for 6 month when stored at 2 – 8 °C, at maximum till expiry of the concentrate. Microbial contamination must be prevented. After the expiry date, the quality guarantee is no longer valid. The aluminium bag containing the microwell strips must be opened in such a way that the clip seal is not torn off. Any microwell strips which are not required must immediately be returned to the aluminium bag and stored at 2 - 8 °C. The

colourless substrate must also be protected from direct light to prevent it from decomposing or turning blue due to auto-oxidation. Once the substrate has turned blue, it must not be used.

6. Additional necessary reagents – and necessary equipment

6.1. Reagents

- Distilled or deionised water

6.2. Accessories

- Microbalance or RIDA®TUBE Calprotectin (Art. No. GZ3016)
- Vortex mixer (optional, see 9.4.)
- Micropipettes for 20 - 100 µl and 1 ml volume
- Measuring cylinder (1000 ml)
- Stop clock
- Microplate washer or multichannel pipette (300 µl)
- Microplate reader (450 nm, reference wavelength ≥ 620 nm)
- Filter paper (laboratory towels)
- Waste container containing 0.5 % hypochlorite solution

7. Precautions for users

For *in vitro* diagnostic use only.

This test must only be carried out by trained laboratory personnel. The guidelines for working in medical laboratories must be followed and the instructions for carrying out the test must be strictly adhered to.

The stool samples must be treated as potentially infectious and handled in accordance with the national safety regulations.

Samples or reagents must not be pipetted by mouth and contact with injured skin or mucous membranes must be prevented. When handling the samples, wear disposable gloves and when the test is finished, wash your hands. Do not smoke, eat or drink in areas where samples or test reagents are being used.

The wash buffer contains 0.1 % Thimerosal as a preservative. This substance must not be allowed to come into contact with skin and mucous membranes.

Calibrator, standards, positive control, low positive control, extraction and sample dilution buffer contain 0.05% resp. 0,1 % NaN_3 as a preservative. This substance must not be allowed to come into contact with the skin or mucous membrane.

Hydrogen peroxide can cause burns. Handle with care.

The stop reagent contains 1 N sulphuric acid. Avoid contact with the skin and clothing. If the skin is contaminated with the reagent, rinse it off with water.

All reagents and materials coming into contact with potentially infectious samples must be treated with suitable disinfectants or autoclaved at 121 °C for at least 1 hour. CAUTION: To prevent the formation of poisonous gases, any liquid waste containing stop reagent must be neutralised before adding to hypochlorite solution.

8. Sample collection and storage

The stool samples should be transported chilled where possible and stored at 2 - 8 °C before testing. If they are not used immediately after arrival (within 3 days after arrival), we recommend storage at -20 °C or lower. By freezing native stool samples neutrophile granulocytes can burst open and release calprotectin. This can result in slightly increased calprotectin concentrations of frozen samples in contrast to fresh samples. Avoid freezing and thawing the sample repeatedly. Stool samples should not be collected in transport containers which contain transport media with preservatives, animal sera, metal ions, oxidising agents or detergents since these may interfere with the RIDASCREEN® Calprotectin test.

9. Test procedure

9.1. General information

All reagents and the microwell plate must be brought to room temperature (20 - 25 °C) before use. The microwell strips must not be removed from the aluminium bag until they have reached room temperature. The reagents must be thoroughly mixed immediately before use. After use, the microwell strips (in sealed bags) and the reagents must be stored at 2 - 8 °C. Once used, the microwell strips must not be used again. The reagents and microwell strips must not be used if the packaging is damaged or the vials are leaking. In order to prevent cross contamination, the samples must be prevented from coming into direct contact with the kit components. The test must not be carried out in direct sunlight. We recommend covering the microwell plate or placing film on it to prevent evaporation losses.

9.2. Preparing the wash buffer

Mix 1 part wash buffer concentrate Wash with 9 parts distilled water (1:10). Pour 100 ml concentrate in a 1000 ml measuring cylinder and stock up with distilled water to 1000 ml. Any crystals present in the concentrate must be dissolved beforehand by warming in a water bath at 37 °C.

9.3. Preparing the extraction buffer

Mix 1 part extraction buffer Extract with 2 parts distilled water (1:3).

9.4. Preparing the samples

The stool samples should be stored continuously if possible at 2 - 8 °C before use. If they are not used immediately after arrival (within 3 days after arrival), we recommend storage at -20 °C or lower. Frozen samples should be slowly adapted to room temperature.

9.4.1. Sample preparation and suspension by weighing

Weigh exactly 100 mg stool sample into a labelled test tube and then add exactly 5 ml diluted extraction buffer using a pipette (1:50 dilution).

Alternatively, between 80 and 130 mg of stool can be weighed in and suspended in a correspondingly reduced or increased volume (see tab. 1) of diluted extraction buffer (in order to make sure that the dilution ratio (1:50) is constant).

Tab.1: Data on the required amounts of diluted extraction buffer depending on the amount of stool weighed in

Weight [mg]	Volume [ml]
80	4.00
85	4.25
90	4.50
95	4.75
100	5.00
105	5.25
110	5.50
115	5.75
120	6.00
125	6.25
130	6.50

When resuspending the stool sample in an invariable volume of 5 ml of extraction buffer, the variable dilution factor has to be taken into account in the evaluation (see tab. 2).

Tab.2: Specification of the dilution factor at invariable addition of diluted extraction buffer (5 ml), depending on the amount of stool weighed in

Weight [mg]	Dilution factor [ml]
80	62.50
85	58.82
90	55.55
95	52.63
100	50.00
105	47.62
110	45.45
115	43.45
120	41.66
125	40.05
130	38.46

The stool suspension is homogenised by thoroughly mixing it on a vortex mixer, regardless of the method used to weigh in the stool sample. If the stool is in liquid form, use a pipette to take exactly 100 µl and suspend it in exactly 5 ml diluted extraction buffer.

After this, samples should incubate at RT for 5 min, then be vortexed thoroughly again.

The homogenised suspension must then be centrifuged at above 3000 g for 10 minutes in order to sediment large stool particles. The supernatant of the extract must be further diluted for immediate use in the assay.

a. Manual sample dilution

Further dilute 20 µl of the clarified supernatant in 980 µl sample dilution buffer Diluent | 3 (1:50). Then use 100 µl finally diluted stool sample for testing.

Alternatively, as a method for sample preparation and homogenization the RIDA®TUBE Calprotectin (Art. No. GZ3016; see 9.4.2.), a stool collection tube, can be used.

b. Sample dilution in an automated system

If the assay is to be run on the DSX™ automated ELISA system (Dynex Technologies, Inc.), the specific assay protocol required for this should be requested from R-Biopharm AG and applied to the system. This system performs automated sample dilution, as described below.

First, 20 µl of clarified supernatant is pipetted into a deep-well plate by the automated ELISA system and diluted with 980 µl of dilution buffer Diluent | 3 (1:50). Two mixing cycles are then performed.

The required number of coated wells is inserted in the microwell holder of the RIDASCREEN® Calprotectin microtiter plate Plate.

100 µl aliquots of the diluted sample are removed from the deep-well plate and transferred to the RIDASCREEN® Calprotectin microwell plate Plate (final dilution: 1:2500).

For instructions on how to perform the assay with other ELISA automated pipetting systems, please contact R-Biopharm AG.

9.4.2. Sample preparation and suspension using RIDA®TUBE Calprotectin (Art. No. GZ3016)

RIDA®TUBE Calprotectin (Art. No. GZ3016) can be used as a quick and clean alternative to the procedure for the preparation and suspension of samples outlined in Section 9.4.1, which requires the weighing of stool samples. These tubes, which are supplied as accessories to the RIDASCREEN® Calprotectin assay, simplify sample preparation considerably.

Each RIDA®TUBE Calprotectin comes pre-filled with 2.5 ml of ready-to-use extraction buffer and accommodates a 10 mg stool sample. When processing liquid stool samples, 10 µl of the stool sample can be measured using the pipette and pipetted directly into the extraction buffer.

The sample collection and extraction procedure is described in detail in the instruction leaflet, which is enclosed in each package of RIDA®TUBE Calprotectin and can be downloaded from www.r-biopharm.de.

Stool extracts must not be stored and should be assayed directly after dilution.

a. Manual sample dilution

100 µl of the stool suspension obtained using the RIDA®TUBE Calprotectin (Art. No. GZ3016) – as described in Section 8 of the instructions for product GZ3016 – is diluted in 900 µl of RIDASCREEN® sample dilution buffer Diluent | 3. 100 µl of this final dilution of the stool sample is directly employed in the assay.

b. Automated sample dilution

If the assay is to be run on the DSX™ automated ELISA system (Dynex Technologies, Inc.), the specific assay protocol required for this should be requested from R-Biopharm AG and applied to the system.

The required number of coated wells is placed in the microwell holder of the RIDASCREEN® Calprotectin microtiter plate Plate.

When using the automated ELISA system, the stool suspension extracted with a RIDA®TUBE Calprotectin is automatically diluted 1:10 (final dilution: 1:2500) on the microwell plate Plate. For this purpose 10 µl of the stool suspension is pipetted directly from the RIDA®TUBE Calprotectin

(Art. No. GZ3016) into the RIDASCREEN® Calprotectin microwell plate and diluted with 90 µl of sample dilution buffer Diluent | 3.

For instructions on how to perform the assay with other ELISA automated pipetting systems, please contact R-Biopharm AG.

9.5. First incubation

After placing a sufficient number of wells in the holder, add 100 µl of calibrator Calibrator (1-point calibration procedure, in duplicate recommended), **or** 100 µl of Standard 1 to Standard 5 (standard curve procedure, in duplicate recommended), of sample dilution buffer Diluent | 3 (= negative control), of positive control Control | + and of the stool suspensions being analysed to the relevant wells. If used, also add 100 µl of the low positive control Low control | + to the relevant wells. Then incubate the plate at room temperature (20 - 25 °C) for 1 hour.

9.6. First washing

Careful washing is important in order to achieve the correct results and should therefore be carried out strictly in accordance with the instructions. The incubated substance in the wells must be emptied into a waste container containing hypochlorite solution for disinfection. Tap the plate upside down vigorously against absorbent paper to ensure complete removal of liquid from the microwells. Then wash the plate 5 times using 300 µl diluted wash buffer each time (see 9.2). Tap the plate upside down vigorously against absorbent paper to ensure complete removal of liquid from the microwells.

When using a microplate washer, make sure that the machine is correctly adjusted to the type of microwell plate being used. Furthermore, a stool suspension which is not completely particle-free before the first washing should be removed manually by centrifuging in order to avoid blocking the wash needles. Also make sure that all of the liquid is sucked away during each washing phase. After washing for the last time, tap the plate upside down vigorously against absorbent paper to ensure complete removal of liquid from the microwells.

9.7. Second incubation

Add 100 µl conjugate Conjugate into each well. Then incubate the plate at room temperature (20 - 25 °C) for 1 hour.

9.8. Second washing

The incubated substance in the wells must be emptied into a waste container containing hypochlorite solution for disinfection. Tap the plate upside down vigorously against absorbent paper to ensure complete removal of liquid from the microwells. Then wash the plate 5 times using 300 µl diluted wash buffer each time. Tap the plate upside down vigorously against absorbent paper to ensure complete removal of liquid from the microwells.

9.9. Third incubation

Add 100 µl substrate SeroSC to each well. Then incubate the plate at room temperature (20 - 25 °C) in the dark for 15 minutes. After this, stop the reaction by adding 50 µl stop reagent Stop to each well.

After mixing carefully (by lightly tapping the side of the plate) measure the absorbance at 450 nm (reference wavelength ≥ 620 nm) in a plate reader.

Notice: Highly positive patient samples can induce black precipitation of the substrate. These samples should be further diluted 1:10 (final dilution of the stool stample: 1:25000) and be reanalysed.

10. Quality control – indications of reagent expiry

For quality control purposes, the calibrator Calibrator (1-point calibration procedure, in duplicate recommended), or each standard 1 to standard 5 Standard 1 – Standard 5 (standard curve procedure, in duplicate recommended) , sample dilution buffer Diluent | 3 as a negative control and positive control Control | + must be used every time the test is carried out.

The test has been carried out correctly if the extinction (OD) of the negative control at 450 nm/620 nm is smaller than 0.05 and the determined extinction (OD) of the calibrator (1-point calibration procedure) is within the lot-specific range given on the data sheet enclosed. Positive control is decisive for the validity of test results when using the calibrator as well as the five standards and should be within the lot-specific range of concentration which is given on the data sheet.

If running RIDASCREEN® Calprotectin on ELISA processors, the measured OD value for the calibrator Calibrator (1-point calibration procedure) can differ from the value given on the lot-specific data sheet depending on the used instrument. Therefore the positive control is decisive for the validity of results and on ELISA processors. The use of the low positive control Low control | + is not mandatory.

If the values differ from those required, if the substrate is turbid or has turned blue before adding to the wells, this may indicate that the reagents have expired. If the stipulated values are not met, the following points must be checked before repeating the test:

- Expiry date of the reagents used
- Functionality of the equipment being used (e.g. calibration)
- Correct test procedure
- Visual inspection of the kit components for contamination or leaks – a substrate solution which has turned blue must not be used.

If the conditions are still not fulfilled after repeating the test, please contact the manufacturer or your local R-Biopharm distributor.

11. Evaluation and interpretation

The concentration of calprotectin in mg/kg stool is determined with the RIDASCREEN® Calprotectin either by 1-point calibrator procedure (see 11.1.) according to the 4-parameter-logistic-log-model (4PL) or by standard curve procedure (see 11.2.).

The evaluation software RIDA®SOFT Win.net is needed to determine the results. RIDA®SOFT Win.net or an update can be obtained from R-Biopharm AG or from your local R-Biopharm distributor.

Another evaluation software which provides the 4-parameter-logistic-log-model can also be used as an alternative to RIDA®SOFT Win.net.

11.1. Quantification by 1-Point Calibration Procedure (4-Parameter-logistic model)

The parameters (A - D) of the standard curve required for the 4PL calculation and the setpoint value of calibrator, positive control and low positive control are listed on the lot-specific datasheet supplied with the kit and must be compared to the values in the evaluation software before each measurement using the corresponding information on this datasheet.

R-Biopharm AG has determined the standard curve (including the parameters A - D) as well as the setpoint values and allowed OD-range of calibrator resp. range of concentration for positive control and low positive control under optimum conditions for each kit lot in the final quality control.

The calibrator is tested in routine in order to compensate test fluctuations and check the quality of each test run. The positive control is decisive for the validity of test results.

A correction factor F is calculated internally by RIDA®SOFT Win.net from the average value of the double determination of calibrator control and its setpoint value and is reconciled with the absorbance for the stool samples. A safe and reliable evaluation of the test results can be carried out within the limits of the standard curve.

11.2. Quantification by Standard Curve Procedure

Evaluation of RIDASCREEN Calprotectin can be achieved by standard curve that must always be processed when running the test. For an exemplary progression of a standard curve, please refer to the attached lot-specific data sheet.

In final quality control, R-Biopharm AG has determined the target values and the allowed range of concentration for the positive control and low-positive control for each kit lot under optimal test conditions. In quantifications by standard curve procedure the positive control is decisive for the validity of the test results. The predefined range of concentration for the positive control must be achieved.

11.3. Test result

For a cut-off value of > 50 mg calprotectin / kg stool, the result must be considered positive. If the results lie below the cut-off value, the samples must be considered negative.
The cut-off-value suggested for adults (50 mg/kg) can be used for children aged from 4 – 17 years as well.

We recommend that a separate standard value range is established by each laboratory.

Samples which show Calprotectin concentrations of more than 600 mg/kg, when using 1-Point Calibration or Standard Curve for quantification are recommended to be further diluted (1:5 in Diluent 3) and reanalysed in the assay.

Samples which show OD-values higher than 3.0, when using 1-Point Calibration for quantification, are recommended to be further diluted (1:5 in Diluent 3) and to be reanalysed in the assay.

12. Limitations of the method

RIDASCREEN® Calprotectin detects epitopes of human Calprotectin in stool samples. Diagnosis should not be made on determining the concentration of calprotectin alone, but should take into consideration clinical history and symptoms.

13. Performance characteristics

13.1. Precision

13.1.1. Intra-Assay-Reproducibility

The intra-assay reproducibility was determined in a single run using 4 references in 40 replicates each. From the OD-values of these measurements calprotectin concentrations have

been determined. The mean value (MV), standard deviation (SD) and coefficient of variation (CV) were calculated for each sample. The results are listed in Table 3.

Tab. 3: Intra-Assay-Reproducibility RIDASCREEN® Calprotectin

	Calibrator			Standard Curve		
	MV (mg/kg)	SD (mg/kg)	**CV (%)**	MV (mg/kg)	SD (mg/kg)	**CV (%)**
Reference A	53.8	2.9	**5.4**	56.6	3.0	**5.3**
Reference B	96.6	5.5	**5.7**	102.4	6.3	**6.2**
Reference C	136.5	5.6	**4.1**	130.5	5.2	**4.0**
Reference D	246.7	14.1	**5.7**	267.6	15.0	**5.6**

13.1.2. Inter-Assay-Reproducibility

The inter-assay reproducibility was determined in duplicate in 20 runs (2 runs a day) using four references by three technicians. From the OD-values of these measurements calprotectin concentrations have been determined. The mean value (MV), standard deviation (SD) and coefficient of variation (CV) were calculated for each sample. The results are listed in Table 4.

Tab. 4: Inter-Assay-Reproducibility RIDASCREEN® Calprotectin

	Calibrator			Standard Curve		
	MV (mg/kg)	SD (mg/kg)	**CV (%)**	MV (mg/kg)	SD (mg/kg)	**CV (%)**
Reference A	55.7	4.3	**7.7**	57.5	4.1	**7.1**
Reference B	106.5	7.2	**6.8**	112.8	8.3	**7.3**
Reference C	129.6	7.8	**6.0**	138.5	7.6	**5.5**
Reference D	260.9	28.3	**10.9**	284.7	22.7	**8.0**

13.2. Extraction Precision

Extraction precision was determined in duplicate in 10 runs (5 days, 2 runs a day) at different days by two technicians. with 3 stool sample (concentrations > 50 mg/kg). The samples have been extracted before each run and diluted as required. From the OD-values of these measurements calprotectin concentrations have been determined. The mean value (MV), standard deviation (SD) and coefficient of variation (CV) were calculated for each sample. The results are listed in Table 5.

Tab. 5: Extraction Precision

	Calibrator			Standard Curve		
	MV (mg/kg)	SD (mg/kg)	**CV (%)**	MV (mg/kg)	SD (mg/kg)	**CV (%)**
Sample A	64.0	5.0	**7.9**	67.4	5.0	**7.5**
Sample B	114.1	10.3	**9.0**	123.2	10.7	**8.7**
Sample C	264.0	33.3	**12.6**	284.8	22.1	**7.8**

14. Literature

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