DRG

DRG® Calprotectin (stool) ELISA (EIA-4498)

CE

RUO in the USA

Revised 28 July 2010 rm (Vers. 8.0)

Please use only the valid version of the package insert provided with the kit.

INTENDED USE

The described Enzyme-Linked-Immuno-Sorbent-Assay (ELISA) Kit is intended for the quantitative determination of Calprotectin (MRP (8/14) in stool.
It is for in vitro diagnostic use only. In the United States, this kit is intended for Research Use Only.

INTRODUCTION

Alternative names:
Calgranulin A: MRP8, S100A8, CP-10 (in mouse)
Calgranulin B: MRP14, S100A9,
MRP8/14: L1, (p8,14), p34
Calprotectin is a calcium-binding protein secreted predominantly by neutrophils and monocytes. Fecal Calprotectin is a marker for neoplasic and inflammatory gastrointestinal diseases.
It is often difficult to distinguish between irritable bowel syndrome and chronic inflammatory bowel disease. This leads in many cases to extensive and unnecessary colonoscopic examinations. The Calprotectin test allows clear differentiation between the two patient groups. Fecal Calprotectin levels correlate significantly with histologic and endoscopic assessment of disease activity in Morbus Crohn's disease and ulcerative colitis as well as with the fecal excretion of indium-111-labelled neutrophilic granulocytes that has been suggested as the "gold standard" of disease activity in inflammatory bowel disease. However, measuring 111-indium-labeled granulocytes is very costly (patient's hospitalization, analysis and disposal of isotopic material) and is connected with radioactive exposition of the patients. For this reason, a repeated application to children and pregnant women is not recommended.
Elevated levels of Calprotectin are a much better predictor of relapse than standard inflammatory markers (CRP, ESR HB). Comparing this marker with standard fecal occult blood screening in colorectal cancer demonstrates clearly the diagnostic advantages of the fecal Calprotectin test. The parameter is of a high diagnostic value: if the Calprotectin level in stool is low, the probability is high that no organic intestinal disease exists.

Indication

- Marker for acute inflammation
- Estimation of gastrointestinal inflammation degree
- Parameter for monitoring Morbus Crohn's disease, Colitis ulcerosa or the patient's status after removal of polyps.
- Discrimination between patients with inflammatory bowel disease (acute Morbus Crohn's disease and ulcerative colitis) and irritable bowel syndrome when using a fecal test system

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MATERIAL SUPPLIED

	Kit Components	Quantity
PLATE	**Microtiter plate**; One holder with precoated strips	12 x 8 wells
WASHBUF	**Wash Buffer**, concentrate 10x	2 x 100 mL
EXBUF	**Extraction Buffer** concentrate 2.5x	2 x 90 mL
AB	**Detections antibody**, (monoclonal anti-Calprotectin (MRP 8/14) antibody, biotinylated), concentrate	50 µL
STD	Calprotectin **Standards**, lyophilized (0; 3.9; 15.6; 62.5; 250 ng/mL)	2 x 5 vials
CTRL	**Control 1**, lyophilized (see specification for range)	2 x 1 vial
CTRL 2	**Control 2**, lyophilized (see specification for range)	2 x 1 vial
CONJ	**Conjugate**, (extravidin peroxidase labeled, concentrate	50 µL
SUB	**TMB Substrate** (Tetramethylbenzidine), ready to use	15 mL
STOP	**Stop Solution**, ready to use	15 mL

MATERIAL REQUIRED BUT NOT SUPPLIED

- Bidistilled water (aqua bidest.)
- Laboratory balance
- Precision pipettors calibrated and tips to deliver 10-1000 µL
- Covering foil for the microtiter plate
- Horizontal microtiter plate shaker with 37 °C incubator
- A multi-channel dispenser or repeating dispenser
- Centrifuge capable of 3000 x g
- Vortex-Mixer
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader at 450 nm (reference wave length 620 or 690 nm)

PREPARATION AND STORAGE OF REAGENTS

To run assay more than once, ensure that reagents are stored at the conditions stated on the label. **Prepare only the appropriate amount necessary for each assay.** The kit can be used up to 4 times within the expiry date stated on the label.

Reagents with a volume less than **100 µL** should be centrifuged before use to avoid loss of volume.

The **ELISA WASHBUF** (wash buffer concentrate) must be diluted with aqua bidist. **1:10** before use (100 ml WASHBUF + 900 ml aqua bidist.), mix well.

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Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at 37°C in a water bath before dilution.
The **buffer concentrate** is stable at **2–8°C** until the expiry date stated on the label.
Diluted **buffer solution** can be stored in a closed flask at **2–8°C for one month.**

The **EXBUF** (extraction buffer concentrate) must be diluted with aqua bidist. **1:2.5** before use
(90 ml EXBUF + 135 ml aqua bidist.), mix well.
Crystals could occur due to high salt concentration in the stock solutions. Before dilution, the crystals must be redissolved at 37°C in a water bath.
The **buffer concentrate** is stable at **2–8°C** until the expiry date stated on the label.
Diluted **buffer solution** can be stored in a closed flask at **2–8°C for three months.**

The lyophilized **STD** (standards) and **CTRL** (controls) are stable at **2–8°C** until the expiry date stated on the label. The **STD** (standards) and **CTRL** (controls) must be reconstituted with **500 µl** aqua bidest. Allow the vial content to dissolve for 10 minutes and mix thoroughly by gentle inversion to insure complete reconstitution. **Reconstituted standards and controls can be stored at 2-8°C for four weeks.**

The **AB** (detection antibody) must be diluted **1:1000** in wash buffer (10 µl AB + 10 ml wash buffer).
The antibody is stable at **2-8 °C** until expiry date given on the label.
Diluted antibody solution is not stable and can not be stored.

The **CONJ** (conjugate) must be diluted **1:1000** in wash buffer (10 µl CONJ + 10 ml wash buffer).
The undiluted conjugate is stable at **2–8°C** until the expiry date stated on the label.
Diluted conjugate is not stable and can not be stored.

All other test reagents are ready to use. The test reagents are stable until the expiry date given on the label when stored **at 2-8°C**.

SAMPLE PREPARATION

Extraction of the stool sample

We recommend the following sample preparation:

1. We recommend the use of a stool sample preparation kit for dosing **100 mg of stool sample** (e.g. Sample preparation kit from Roche Diagnostics, Mannheim, Germany; cat # 745804). The stool sample must be suspended in **5 mL** extraction buffer.

   Constant buffer volume: 5 mL

   Constant dilution factor: 1:50

2. Alternatively, stool samples can be manually weighted within the range of 80 – 120 mg. Please note the exact sample amount!

   a. Add **5 mL** buffer to the stool sample not depending on the sample amount.

   Constant buffer volume: 5 mL

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The dilution factor varies depending on the sample amount which has to be considered in the subsequent calculations. Please have a look at the table for the correction factors below:

Weight [mg]	Dilution factor
80	62.5
82	60.9
84	59.5
86	58.1
88	56.8
90	55.6
92	54.3
94	53.2
96	52.1
98	51.0
100	**50**

Weight [mg]	Dilution factor
102	49.0
104	48.1
106	47.2
108	46.3
110	45.5
112	44.6
114	43.9
116	43.1
118	42.4
120	41.6

b. **The buffer volume** for the individual samples varies depending on the sample amount (see table). The dilution factor remains constant.

Variable buffer volume

Constant dilution factor: 1:50

Therefore, the same dilution factor can be used for all samples in the subsequent evaluation of the results.

Weight [mg]	Buffer Volume [mL]
80	4.0
82	4.1
84	4.2
86	4.3
88	4.4
90	4.5
92	4.6
94	4.7
96	4.8
98	4.9
100	**5.0**

Weight [mg]	Buffer Volume [mL]
102	5.1
104	5.2
106	5.3
108	5.4
110	5.5
112	5.6
114	5.7
116	5.8
118	5.9
120	6.0

Afterwards, mix stool sample and buffer; vortex for at least 30 sec. depending on the stool consistency.
Transfer ca. 1 mL stool suspension (**dilution step I**) to an Eppendorf-tube and centrifuge for 5 minutes at 13000 g.

Dilution of samples

Stool samples

The supernatant of the extraction (dilution step I) is diluted **1:50 with wash buffer**.
For example:
20 µL supernatant (dilution I) + 980 µL wash buffer = 1:50 (**dilution step II**)
For analysis, pipette **100 µL** of the supernatant of **dilution step II** per well.
Calprotectin in stool is described to be stable for approximately 6 days. Nevertheless, we recommend to store the samples for not more than 48 h at 2-8 °C. Long term storage is recommended at -20 °C. Allow frozen samples to thaw slowly, preferably at 2–8° C over night and warm the samples to room temperature before analysis.
(Poullis A et al. (2002) Aliment Pharmacol Thr 16:675-681)

ASSAY PROCEDURE

Principle of the test

The assay utilizes the two-site "sandwich" technique with two selected monoclonal antibodies that bind to human Calprotectin.
Standards, controls and diluted patient samples which are assayed for human Calprotectin are added to wells of microplate coated with a high affine monoclonal anti-human Calprotectin antibody.
During the first incubation step, Calprotectin in the samples is bound by the immobilized antibody. In a next incubation step, a biotinylated monoclonal anti-human Calprotectin antibody is added to each microtiter well. Then a peroxidase labeled extravidin conjugate is added to each well and the following complex is formed: capture antibody - human Calprotectin – biotinylated detection antibody - Peroxidase conjugate.
Tetramethylbenzidine (TMB) is used as a substrate for peroxidase. Finally, an acidic stop solution is added to terminate the reaction. The color changes from blue to yellow.
The intensity of the yellow color is directly proportional to the Calprotectin concentration of sample.
A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration is generated, using the values obtained from standard. Calprotectin present in the patient samples, is determined directly from this curve.

Test procedure

1. Bring all reagents and samples to room temperature (18-26 °C) and mix well
2. Mark the **positions of STD /SAMPLE/CTRL** (Standards/Sample/Controls) in duplicate on a protocol sheet
3. **Take** as many **microtiter strips as needed** from kit. Store unused strips covered at 2-8° C. Strips are stable until expiry date stated on the label
4. Wash each well **5 times** with **250 µl of diluted wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper
5. Add **100 µl of STD/SAMPLE/CTRL** (Standard/Sample/Controls) in duplicate into respective well
6. Cover plate tightly and **incubate for 1 hour at 37 °C** on a horizontal mixer**
7. Aspirate the contents of each well. Wash each well **5 times** with **250 µl of diluted wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper
8. Add **100 µl AB** (detection antibody) into each well

9. Cover plate tightly and **incubate for 1 hour at 37 °C** on a horizontal mixer**
10. Aspirate the contents of each well. Wash each well **5 times** with **250 µl of diluted wash buffer.** After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper
11. Add **100 µl CONJ** (conjugate) into each well
12. Cover plate tightly and **incubate for 1 hour at 37 °C** on a horizontal mixer**
13. Aspirate the contents of each well. Wash each well **5 times** with **250 µl of diluted wash buffer.** After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper
14. Add **100 µl of SUB** (substrate) into each well
15. Incubate for **10 - 20 minutes at room temperature** (18-26°C) in the dark*
16. Add **50 µl of STOP** (stop solution) into each well, mix thoroughly
17. Determine **absorption immediately** with an ELISA reader at **450 nm** against 620 nm (or 690 nm) as a reference. If no reference wavelength is available, read only at 450 nm. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at 405 nm against 620 nm as a reference

*The intensity of the color change is temperature sensitive. We recommend to observe the procedure of the color change and to stop the reaction upon good differentiation.

**The above incubation steps at 37 °C on a horizontal mixer are recommended by the producer. If there is no possibility to incubate at 37 °C, while shaking, we recommend to incubate at 37 °C without any shaking.

RESULTS

The following algorithms can be used alternatively to calculate the results. We recommend to use the "4-Parameter-algorithm".

1. 4-parameter-algorithm
   It is recommended to use a linear ordinate for optical density and a logarithmic abscissa for concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e. g. 0.01).
2. Point-to-point-calculation
   We recommend a linear ordinate for optical density and a linear abscissa for concentration.
3. Spline-algorithm
   We recommend a linear ordinate for optical density and a logarithmic abscissa for concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e. g. 0.01).
   The plausibility of the pairs of values should be examined before the automatic evaluation of the results. If this option is not available with the used program, a control of the paired values should be done manually.

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Stool samples
To obtain the calprotectin concentration in stool samples, multiply the estimated value by the dilution factor according to the sample preparation:

Sample preparation 1 and 2b: dilution factor constant : 1 : 50
Multiply the obtained result by **2500** (dilution step I x dilution step II) to get the final concentration.

Sample preparation 2a: dilution factor is variable.
The corresponding dilution factor is taken from the table and should be multiplied by 50 (dilution step II) to get the final concentration.

LIMITATIONS

Stool samples with Calprotectin levels greater than the highest standard value, should be diluted with wash buffer, and be re-assayed.

QUALITY CONTROL

Control samples should be analyzed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid, if within the same assay one or more values of the quality control sample are outside the acceptable limits.

Expected values
Normal ranges
(1 g stool is equivalent to 1 mL)
Calprotectin in stool: < 15 mg/L
Grey area: 10 - 15 mg/L
We recommend each laboratory to establish its own norm concentration range.

Performance Characteristics

Precision and reproducibility

Intra-Assay (n=40)		
Sample	**Calprotectin [ng/mL]**	**CV [%]**
1	12.768	9.75
2	18.997	4.18

Intra-Assay (n=20)		
Sample	**Calprotectin [ng/mL]**	**CV [%]**
1	8.485	16.55
2	17.512	6.04

Recovery

Two samples were spiked with 4 different Calprotectin standards and measured using this assay. n=2

Sample [ng/mL]	Spike [ng/mL]	Calprotectin expected [ng/mL]	Calprotectin measured [ng/mL]
8.2	50.0	58.2	65.0
8.2	21.0	29.2	34.0
8.2	9.5	17.7	20.0
8.2	4.5	12.7	15.0
19.2	50.0	69.2	77.3
19.2	21.0	40.2	41.5
19.2	9.5	28.7	29.9
19.2	4.5	23.7	25.2

Sensitivity

The sensitivity was set as B_0 + 1 SD. The zero-standard was measured 24 times.

Sample	Calprotectin mean value [OD]	Standard variation (SD)	Detection limit [ng/mL]
1	0.094	0.011	2.915

Cross-reactivity

No cross-reactivity was observed to the following plasma proteins:
Lysozyme - 0%
PMN-Elastase - 0%
Myeloperoxidase - 0%
Lactoferrin - 0%

Linearity

Two patient samples were diluted with wash buffer and analyzed. The results are shown below: n=2

Sample	Dilution	Expected [ng/mL]	Measured [ng/mL]
A	10 000	223.0	223.0
	20 000	111.5	116.5
	40 000	55.75	61.0
B	10 000	135.0	137.0
	20 000	67.5	62.5
	40 000	33.75	36.125

PRECAUTIONS

- For in vitro diagnostic use only. In the United States, this kit is intended for Research Use Only.
- Quality control guidelines should be observed.
- Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
- Kit reagents contain sodium azide or thimerosal as bactericides. Sodium azide and thimerosal are toxic. Substrates for the enzymatic color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- Stop solution is composed of sulfuric acid, which is a strong acid. Even diluted, it still must be handled with care. It can cause acid burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spills should be wiped out immediately with copious quantities of water.

TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay.
- Reagents should not be used beyond the expiration date shown on the kit label.
- Substrate solution should remain colourless until use.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents.
- The assay should always be performed according the enclosed manual.

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GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and put on the market according to the IVD guidelines of 98/79/EC.
- All reagents in the kit package are for in vitro diagnostic use only. In the United States, this kit is intended for Research Use Only.
- Guidelines for medical laboratories should be observed.
- Incubation time, incubation temperature and pipetting volumes of the components are defined by the producer. Any variation of the test procedure, which is not coordinated with the producer, may influence the results of the test. DRG can therefore not be held responsible for any damage resulting from wrong use.
- Warranty claims and complaints in respect of deficiencies must be logged within 14 days after receipt of the product. The product shall be send to DRG together with a written complaint.

REFERENCES / Literature / Literatur / Bibliografia

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5. Langhorst J, Koelzer J, Elsenbruch S, Rueffer A, Michalsen A, Dobos GJ (2007) Non-invasive Marker der Entzündungsaktivität bei Patienten mit chronisch entzündlichen Darmerkrankungen (CED): Vergleich von Lactoferrin, Calprotectin, PMN-Elastase im Stuhl, Serum-CRP und klinischen Aktivitätsindizes. Z Gastroenterol 45: P261
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Symbols used with DRG Assays

Symbol	English	Deutsch	Français	Español	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
CE	European Conformity	CE-Konfirmitäts-kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Vertreiber	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità