

PhiCal[®] Calprotectin ELISA Kit

*For the in vitro determination of
calprotectin (MRP 8/14, S100A8/A9) in stool*

[**PhiCal[®]**: registered German trademark of Immundiagnostik AG]

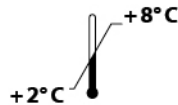
EU: IVD / CE

US: Research Use Only. Not for use in diagnostic procedures.

Valid from 23.01.2014



K 6927



Immundiagnostik AG

Table of Contents

1. INTENDED USE	19
2. CLINICAL RELEVANCE	19
3. MATERIAL SUPPLIED	20
4. MATERIAL REQUIRED BUT NOT SUPPLIED	20
5. PREPARATION AND STORAGE OF REAGENTS	21
6. SPECIMEN COLLECTION AND PREPARATION	22
<i>Sample stability and storage</i>	22
<i>Extraction of the stool sample</i>	22
<i>Dilution of samples</i>	23
7. ASSAY PROCEDURE	23
<i>Principle of the Test</i>	23
<i>Test procedure</i>	24
8. RESULTS	25
9. LIMITATIONS	25
10. QUALITY CONTROL	26
<i>Reference range</i>	26
<i>Reference ranges for fecal calprotectin in children</i>	26
11. PERFORMANCE CHARACTERISTICS	27
<i>Precision and reproducibility</i>	27
<i>Specificity</i>	28
<i>Dilution recovery</i>	28
<i>Analytical Sensitivity</i>	29
<i>Spiking Recovery</i>	29
12. PRECAUTIONS	29
13. TECHNICAL HINTS	30
14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE	30
15. REFERENCES	31
<i>General literature</i>	31
<i>Publications using Immundiagnostik Calprotectin ELISA</i>	31

1. INTENDED USE

The described **PhiCal**® Calprotectin ELISA is intended for the quantitative determination of calprotectin (MRP (8/14, S100A8/A9) in stool. For *in vitro* diagnostic use only.

2. CLINICAL RELEVANCE

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 neoplastic 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.

Indications

- 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

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity
K 6927MTP	PLATE	Holder with precoated strips	12 x 8 wells
K 6927WP	WASHBUF	ELISA wash buffer concentrate 10x	2 x 100 ml
K 6927EP	EXBUF	Extraction buffer concentrate 2.5x	1 x 100 ml
K 6927PV	SAMPLEBUF	Sample dilution buffer, ready to use	1 x 100 ml
K 6927ST	STD	Calprotectin standards, lyophilized (0; 13; 52; 210; 840 ng/ml)	2 x 5 vials
K 6927KO1	CTRL	Control, lyophilized (see specification for range)	2 x 1 vial
K 6927KO2	CTRL	Control, lyophilized (see specification for range)	2 x 1 vial
K 6927K	CONJ	Conjugate, ready to use	15 ml
K 6927TMB	SUB	TMB substrate (Tetramethylbenzidine), ready to use	15 ml
K 6927AC	STOP	ELISA stop solution, ready to use	15 ml

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultra pure water*
- Laboratory balance
- Calibrated precision pipettors and 10–1000 µl tips
- Foil to cover the microtiter plate
- Multi-channel pipets or repeater pipets
- Centrifuge, 3000 g
- Vortex
- Standard laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader

* Immundiagnostik AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 µm) with an electrical conductivity of 0.055 µS/cm at 25 °C (≤ 18.2 MΩ cm).

5. PREPARATION AND STORAGE OF REAGENTS

- To run assay more than once, ensure that reagents are stored at conditions stated on the label. **Prepare only the appropriate amount necessary for each run.** 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 wash buffer concentrate** (WASHBUF) should be diluted **1:10 in ultra pure water** before use (100 ml concentrate + 900 ml ultra pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. The crystals must be redissolved at room temperature or at 37°C before dilution of the buffer solutions. The **buffer concentrate** is stable at **2-8°C** until the expiry date stated on the label. **Diluted buffer solution (wash buffer)** can be stored in a closed flask at **2-8°C for one month**.
- The **EXBUF** (extraction buffer concentrate) must be diluted with ultra pure water **1:2.5** before use (100 ml EXBUF + 150 ml ultra pure water), 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 of ultra pure water**. Allow the vial content to dissolve for 10 minutes at room temperature, and mix thoroughly by gentle inversion to insure complete reconstitution. **Reconstituted standards and controls can be stored at 2-8°C for 4 weeks**.
- All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at **2-8°C**.

6. SPECIMEN COLLECTION AND PREPARATION

Sample stability and storage

Raw stool

Calprotectin in stool is described to be stable for at least 3 days at room temperature (Tøn et al. (2000) Clin Chim Acta). Nevertheless, we recommend storing the samples for no 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, and warm the samples to room temperature before analysis. Avoid repeated freezing and thawing of the sample. Freezing can cause neutrophil granulocytes in the stool sample to burst and release calprotectin. Therefore frozen samples can be expected to contain slightly elevated concentrations of calprotectin compared to fresh samples.

Chemical or biological additives in stool sample tubes may interfere with PhiCal® Calprotectin. Therefore use only empty tubes or tubes filled with the extraction buffer supplied by Immundiagnostik.

Stool extracts

Stool extract without sediment is stable for nine days at room temperature, 2–8 °C and -20 °C.

Extraction of the stool sample

Diluted extraction buffer is used as a sample extraction buffer. We recommend the following sample preparation:

Stool Sample Application System (SAS) (Cat. No.: K 6998SAS)

Stool sample tube – Instructions for use

Please note that the dilution factor of the final stool suspension depends on the amount of stool sample used and the volume of the buffer.

SAS with 1.5 ml buffer:

Applied amount of stool:	15 mg
Buffer Volume:	1.5 ml
Dilution Factor:	1:100

Please follow the instructions for the preparation of stool samples using the SAS as follows:

- The raw stool sample has to be thawed. For particularly heterogeneous samples we recommend a mechanical homogenisation using an applicator, ino-

cultation loop or similar device.

- b) Fill the **empty sample tube** with **1.5 ml** of ready-to-use extraction buffer before using it with the sample. Important: Allow the extraction buffer to reach room temperature.
- c) Unscrew the tube (yellow part of cap) to open. Insert yellow dipstick into sample. The lower part of the dipstick has notches which need to be covered completely with stool after inserting it into the sample. Place dipstick back into the tube. When putting the stick back into the tube, excess material will be stripped off and leave 15 mg of sample to be diluted. Screw tightly to close the tube.
- d) Shake the tube well until no stool sample remains in the notches. Important: Please make sure that you have a maximally homogenous suspension after shaking. Especially with more solid samples, soaking the sample in the tube with buffer for app. 10 minutes improves the result.
- e) Allow sample to stand for app. 10 minutes until sediment has settled. Floating material like shells of grains can be neglected.
- f) Carefully unscrew the complete cap of the tube including the turquoise ring plus the dipstick. Discard cap and dipstick. Make sure that the sediment will not be dispersed again.

Dilution I: 1:100

Dilution of samples

The suspension of the sample preparation procedure (dilution I) is diluted **1:25 with SAMPLEBUF** (sample dilution buffer). For example:

40 µl suspension (dilution I) + **960 µl** SAMPLEBUF = **1:25 (dilution II)**

This results in a final dilution of 1:2500.

For analysis, pipet **100 µl** of **dilution II** per well.

7. 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. Then a peroxidase labeled conjugate is added to each well and the following complex is formed: capture antibody - human calprotectin – 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) vs. concentration is generated, using the values obtained from standard. Calprotectin present in the patient samples, is determined directly from this curve.

Test procedure

We recommend to carry out the tests in duplicate.

1.	Bring all reagents and samples to room temperature (15 - 30°C) and mix well
2.	Mark the positions of STD / SAMPLE / CTRL (standard / samples / controls) 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.	Add 100 µl of STD / SAMPLE / CTRL into respective well
5.	Cover plate tightly and incubate for 30 minutes at room temperature (15 - 30°C)
6.	Aspirate the contents of each well. Wash each well 5 x with 250 µl of wash buffer . After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper
7.	Add 100 µl CONJ (conjugate) into each well
8.	Cover plate tightly and incubate for 30 minutes at room temperature (15 - 30°C)
9.	Aspirate the contents of each well. Wash each well 5 x with 250 µl of wash buffer . After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper
10.	Add 100 µl of SUB (substrate) into each well
11.	Incubate for 10-20 minutes at room temperature (15 - 30°C) in the dark*

12.	Add 100 µl of STOP (stop solution) into each well, mix thoroughly
13.	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 color change and to stop the reaction upon good differentiation.

8. RESULTS

The following algorithms can be used alternatively to calculate the results. We recommend using the „4 parameter algorithm“.

1. 4 parameter algorithm

It is recommended to use a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e.g. 0.001).

2. Point-to-point calculation

We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

3. Spline algorithm

We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

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.

Stool samples

The obtained calprotectin levels of the stool samples have to be multiplied with the dilution factor of 2500 (dilution I x dilution II).

9. LIMITATIONS

Samples with an OD greater than the OD of the highest calibrator should be further diluted with SAMPLEBUF and re-assayed.

10. QUALITY CONTROL

Immundiagnostik recommends the use of external controls for internal quality control, if possible.

Control samples should be analysed 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.

Reference range

1 g stool is equivalent to 1 ml.

- The median value in healthy adults is about 25 mg/kg.
- Samples giving values above 50 mg/kg are regarded as positive.

We recommend each laboratory to establish its own reference concentration range.

Note: Many confounding factors can cause increased levels of fecal calprotectin in the absence of IBD or IBD in a quiescent disease phase, e.g. use of NSAIDs (non steroidal anti inflammatory drugs), any intercurrent gastrointestinal infection, and the presence of malignancies. These factors should be considered in the interpretation of the test results and therapy of IBD. *

*D'Haens et al. *Inflamm Bowel Dis*, 2012; van Rheeën et al. *BMJ*, 2010

Reference ranges for fecal calprotectin in children

Hestvik E et al. (2011) *BMC Pediatrics* **11**:9 doi:10.1186/1471-2431-11-9

Method: 302 apparently healthy children, age 0–12 years, in Kampala, Uganda, were tested for faecal Calprotectin concentration.

Table 1: Faecal Calprotectin concentration in apparently healthy children by age.

Age	Number	Median faecal calprotectin [mg/kg]
0–3 months	14 (4.6)	345 (195–621)
3–6 months	13 (4.3)	278 (85–988)
6–12 months	27 (8.9)	183 (109–418)
1–4 years	89 (29.5)	75 (53–119)
4–12 years	159 (52.6)	28 (25–35)

Conclusion: Concentrations of faecal Calprotectin among healthy children in

Uganda are comparable to those in healthy children living in high-income countries. In healthy infants faecal Calprotectin is high; in children older than 4 years faecal Calprotectin concentration is low.

Fagerberg UL et al. (2003) *J Pediatr Gastroenterol Nutr* **37**:468-472

Method: 117 healthy children age 4–17 years were tested for faecal Calprotectin concentration.

Table 2: Faecal Calprotectin concentration in healthy children by age.

Age	Number	Median faecal calprotectin [mg/kg]
4–6 years	27	28.2
7–10 years	30	13.5
11–14 years	27	9.9
15–17 years	33	14.6

Conclusion: The suggested cut-off level for adults (< 50 mg/kg) can be used for children aged 4–17 years.

11. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Intra-Assay (n = 20)

Sample	Calprotectin [mg/l]	CV [%]
1	89.2	5.6
2	229.1	3.2

Inter-Assay (n = 12)

Sample	Calprotectin [mg/l]	CV [%]
1	107.8	4.4
2	476.1	8.9

Specificity

No cross reactivity was observed to the following plasma proteins:

- Lysozyme 0%
- PMN-Elastase 0%
- Myeloperoxidase 0%
- Lactoferrin 0%

Dilution recovery

Three patient samples were diluted with **SAMPLEBUF** and analyzed. The results are shown below (n = 3):

Sample	Dilution	Calprotectin expected [mg/l]	Calprotectin measured [mg/l]
A	1:2500		820
	1:5000	410	425
	1:10000	205	192
B	1:2500		1120
	1:5000	560	561
	1:10000	280	266.5
C	1:1250		643.0
	1:2500	321.5	344.0
	1:5000	160.8	157.8
	1:10000	80.4	80.0
	1:20000	40.2	39.7
	1:40000	20.1	17.6
	1:80000	10.0	9.5
	1:160000	5.0	5.0

Analytical Sensitivity

The detection limit was set as $B_0 + 6 \text{ SD}$. The Zero-standard was measured 22 times. The values were estimated in relation to the concentration of the calibration curve and resulted in

- a detection limit of 2.099 ng/ml without consideration of the sample dilution factor
- a detection limit of 5.2475 mg/l with consideration of the sample dilution factor

Spiking Recovery

Two samples were spiked with three different calprotectin concentrations and measured using this assay. (n = 2)

Sample	Unspiked Sample [µg/ml]	Spike [µg/ml]	Calprotectin expected [ng/ml]	Calprotectin measured [ng/ml]
1	18	10.5	28.5	29.6
	18	17.5	35.5	35.6
	18	40.5	58.5	59.3
	18	63.3	81.3	83.2
	18	173.2	191.2	188.7
2	20.7	10.5	31.2	33.3
	20.7	17.5	38.2	41.0
	20.7	40.5	61.2	62.7
	20.7	63.3	84.0	90.8

12. PRECAUTIONS

- All reagents in the kit package are for *in vitro* diagnostic use only.
- Control samples should be analyzed with each run.
- 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 Proclin as bactericides. Sodium azide and Proclin are toxic. Substrates for the enzymatic color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- The stop solution consists of diluted sulphuric acid, a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped out immediately with copious quantities of water. Do not breath vapour and avoid inhalation.

13. TECHNICAL HINTS

- Do not interchange different lot numbers of any kit component within the same assay. Furthermore we recommend not to assemble wells of different microtiter plates for analysis, even if they are of the same batch as wells from already opened microtiter plates are exposed to different conditions as sealed ones.
- Reagents should not be used beyond the expiration date stated on 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.

14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE

- This assay was produced and distributed according to the IVD guidelines of 98/79/EC.
- Quality control guidelines should be followed.
- 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. Immundiagnostik AG 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 should be send to Immundiagnostik AG along with a written complaint.

15. REFERENCES

General literature

1. D'Haens, G. et al. Fecal calprotectin is a surrogate marker for endoscopic lesions in inflammatory bowel disease. *Inflammatory bowel diseases* **18**, 2218–24 (2012).
2. Fagerberg, U. L., Lööf, L., Merzoug, R. D., Hansson, L.-O. & Finkel, Y. Fecal calprotectin levels in healthy children studied with an improved assay. *Journal of pediatric gastroenterology and nutrition* **37**, 468–72 (2003).
3. Fagerhol, M. K. Calprotectin, a faecal marker of organic gastrointestinal abnormality. *Lancet* **356**, 1783–4 (2000).
4. Hestvik, E. et al. Faecal calprotectin concentrations in apparently healthy children aged 0-12 years in urban Kampala, Uganda: a community-based survey. *BMC pediatrics* **11**, 9 (2011).
5. Poullis, A., Foster, R., Northfield, T. C. & Mendall, M. A. Review article: faecal markers in the assessment of activity in inflammatory bowel disease. *Alimentary pharmacology & therapeutics* **16**, 675–81 (2002).
6. Tibble, J. et al. A simple method for assessing intestinal inflammation in Crohn's disease. *Gut* **47**, 506–13 (2000).
7. Tibble, J. A., Sigthorsson, G., Bridger, S., Fagerhol, M. K. & Bjarnason, I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Gastroenterology* **119**, 15–22 (2000).
8. Tøn, H. et al. Improved assay for fecal calprotectin. *Clinica chimica acta; international journal of clinical chemistry* **292**, 41–54 (2000).
9. van Rheenen, P. F., Van de Vijver, E. & Fidler, V. Faecal calprotectin for screening of patients with suspected inflammatory bowel disease: diagnostic meta-analysis. *BMJ (Clinical research ed.)* **341**, c3369 (2010).

Publications using Immundiagnostik Calprotectin ELISA

10. Chen, C.-C., Huang, J.-L., Chang, C.-J. & Kong, M.-S. Fecal calprotectin as a correlative marker in clinical severity of infectious diarrhea and usefulness in evaluating bacterial or viral pathogens in children. *Journal of pediatric gastroenterology and nutrition* **55**, 541–7 (2012).
11. Langhorst, J. et al. 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. *Zeitschrift für Gastroenterologie* **45**, P261 (2007).

12. Schröder, O., Naumann, M., Shastri, Y., Povse, N. & Stein, J. Prospective evaluation of faecal neutrophil-derived proteins in identifying intestinal inflammation: combination of parameters does not improve diagnostic accuracy of calprotectin. *Alimentary pharmacology & therapeutics* **26**, 1035–42 (2007).

Used symbols:

Temperature limitation



Catalogue Number



In Vitro Diagnostic Medical Device



Contains sufficient for <n> tests



Manufacturer



Use by



Lot number