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Manual

IDK[®] Calprotectin ELISA

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

Valid from 2024-08-27

REF

K 6967

REF

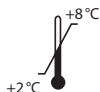
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Table of Contents

1. INTENDED USE	23
2. CLINICAL RELEVANCE	23
3. MATERIAL SUPPLIED	24
4. MATERIAL REQUIRED BUT NOT SUPPLIED	25
5. PREPARATION AND STORAGE OF REAGENTS	25
6. STORAGE AND PREPARATION OF SAMPLES	26
<i>Sample stability and storage</i>	26
<i>Extraction of the stool samples</i>	26
<i>Dilution of samples</i>	27
7. ASSAY PROCEDURE	28
<i>Principle of the test</i>	28
<i>Test procedure</i>	28
8. RESULTS	29
9. LIMITATIONS	30
10. QUALITY CONTROL	30
<i>Reference range</i>	30
<i>Reference ranges for faecal calprotectin in children</i>	31
11. PERFORMANCE CHARACTERISTICS	32
<i>Accuracy – Precision</i>	32
<i>Analytical Sensitivity</i>	33
<i>Accuracy – Trueness</i>	33
<i>Linearity</i>	33
<i>Traceability</i>	35
<i>Analytical specificity</i>	35
12. PRECAUTIONS	36
13. TECHNICAL HINTS	36
14. GENERAL NOTES ON THE TEST AND TEST PROCEDURE	37
15. REFERENCES	37
<i>General literature</i>	37
<i>Publications using IDK® Calprotectin ELISA</i>	38

1. INTENDED USE

This Immundiagnostik AG assay is an enzyme immunoassay 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. Faecalcalprotectin 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. Faecal calprotectin levels correlate significantly with histologic and endoscopic assessment of disease activity in Crohn's disease and ulcerative colitis as well as with the faecal 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-labelled granulocytes is very costly (patient's hospitalisation, 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 faecal occult blood screening in colorectal cancer demonstrates clearly the diagnostic advantages of the faecal 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 Crohn's disease, Colitis ulcerosa or the patient's status after removal of polyps.
- Discrimination between patients with inflammatory bowel disease (acute Crohn's disease and ulcerative colitis) and irritable bowel syndrome when using a faecal test system

3. MATERIAL SUPPLIED

Cat. No.	Label	Kit components	Quantity for cat. no.	
			K 6967	K 6967.20
K 6967	PLATE	Microtiter plate, pre-coated	12 x 8 wells	20 x 12 x 8 wells
K 0001.C.100	WASHBUF	Wash buffer concentrate, 10x	2 x 100 ml	20 x 100 ml
K 6999.C.100	IDK Extract®	Extraction buffer concentrate <i>IDK Extract®</i> , 2.5x	1 x 100 ml	-
K 6967	SAMPLEBUF	Sample dilution buffer, ready-to-use	1 x 100 ml	20 x 100 ml
K 6967	CAL	Calprotectin calibrator, ready-to-use	1 x 1 vial	20 x 1 vial
K 6967	CTRL 1	Control, ready-to-use (see specification for range)	1 x 1 vial	20 x 1 vial
K 6967	CTRL 2	Control, ready-to-use (see specification for range)	1 x 1 vial	20 x 1 vial
K 6967	CONJ	Conjugate, ready-to-use	1 x 15 ml	20 x 15 ml
K 0002.15	SUB	Substrate (Tetramethyl- benzidine), ready-to-use	1 x 15 ml	20 x 15 ml
K 0003.15	STOP	Stop solution, ready-to-use	1 x 15 ml	20 x 15 ml

For reorders of single components, use the catalogue number followed by the label as product number.

4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultrapure water*
- Laboratory balance
- Calibrated precision pipettors and 10–1 000 µl single-use tips
- Foil to cover the microtiter plate
- Multi-channel pipets or repeater pipets
- Centrifuge, 3 000 g
- Vortex
- Standard single-use 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 the 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.
- **Preparation of the wash buffer:** The **wash buffer concentrate (WASHBUF)** has to be diluted with ultra pure water **1:10** before use (100 ml WASHBUF + 900 ml ultra pure water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved at room temperature or in a water bath at 37 °C. The **WASHBUF** is stable at **2–8 °C** until the expiry date stated on the label. **Wash buffer** (1:10 diluted WASHBUF) can be stored in a closed flask at **2–8 °C for 1 month**.
- Use **100 µl** of **sample dilution buffer (SAMPLEBUF)** as **blank (BLANK)**. Pipet into the respective well.
- **Preparation of the extraction buffer:** The **extraction buffer concentrate IDK Extract®** has to be diluted with ultra pure water **1:2.5** before use (100 ml *IDK Extract®* + 150 ml ultra pure water), mix well. Crystals could occur due to high salt concentration in the concentrate. Before dilution, the crystals have to be redissolved at 37 °C in a water bath. The *IDK Extract®* is stable at **2–8 °C** until the expiry date stated on the label. Extraction buffer (1:2.5 diluted *IDK Extract®*) can be stored in a closed flask at **2–8 °C for 4 months**.
- All other test reagents are ready-to-use. Test reagents are stable until the expiry date (see label) when stored at **2–8 °C**.

6. STORAGE AND PREPARATION OF SAMPLES

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 and transporting the samples at room temperature for maximum 2 days. Long term storage up to 12 months 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 IDK® Calprotectin. Therefore use only empty tubes or tubes filled with the extraction buffer IDK Extract® supplied by Immundiagnostik AG.

Stool extracts

Stool extract is stable for nine days at room temperature, 2–8 °C or -20 °C. Avoid more than three freeze-thaw cycles.

Extraction of the stool samples

Extraction buffer (1:2.5 diluted IDK Extract®) 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 sample extraction 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:

- a) The raw stool sample has to be thawed. For particularly heterogeneous samples we recommend a mechanical homogenisation using an applicator, inoculation loop or similar device.
- b) Fill the **empty stool sample tube** with **1.5 ml sample extraction buffer** (1:2.5 diluted *IDK Extract*®) before using it with the sample. **Important:** Allow the sample extraction buffer to reach room temperature.
- c) Unscrew the tube (yellow part of cap) to open. Insert the yellow dipstick into the 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, leaving 15 mg of sample to be diluted. Screw tightly to close the tube.
- d) Vortex 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 sample extraction buffer for ~ 10 minutes improves the result.
- e) Allow sample to stand for ~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 blue 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 sample dilution buffer** (SAMPLEBUF). For example:

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

This results in a final dilution of 1:2 500.

100 µl of **dilution II** per well are used in the test.

7. ASSAY PROCEDURE

Principle of the test

This ELISA is designed for the quantitative determination of calprotectin.

The assay utilises the two-site sandwich technique with two selected monoclonal antibodies that bind to human calprotectin.

Calibrator, 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 immobilised antibody. Then a peroxidase labelled 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 colour changes from blue to yellow. The intensity of the yellow colour is directly proportional to the calprotectin concentration of the sample. The concentration of calprotectin can be quantified by referring the optical density of the calibrator to a lot-dependent master calibration curve.

Test procedure

Bring all **reagents and samples to room temperature** (15–30°C) and mix well.

Mark the positions of calibrator/controls/blank/samples on a protocol sheet.

Take as many microtiter strips as needed from the kit. Store unused strips together with the desiccant bag in the closed aluminium packaging at 2–8°C. Strips are stable until expiry date stated on the label.

For automated ELISA processors, the given protocol may need to be adjusted according to the specific features of the respective automated platform. For further details please contact your supplier or Immundiagnostik AG.

We recommend to carry out the tests in duplicate.

1.	Add each 100 µl calibrator/controls/blank (SAMPLEBUF)/diluted samples into the respective wells.
2.	Cover the strips and incubate for 30 minutes at room temperature (15–30°C) .
3.	Discard the content of each well and wash 5 times with 250 µl wash buffer . After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
4.	Add 100 µl conjugate (CONJ) into each well.

5.	Cover the strips and incubate for 30 minutes at room temperature (15–30°C).
6.	Discard the content of each well and wash 5 times with 250 µl wash buffer . After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.
7.	Add 100 µl substrate (SUB) into each well.
8.	Incubate for 10–20 minutes at room temperature (15–30°C) in the dark* .
9.	Add 100 µl stop solution (STOP) into each well and mix well.
10.	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 colour change is temperature sensitive. We recommend to observe the colour change and to stop the reaction upon good differentiation.

8. RESULTS

For result evaluation, please use a four parametric logit-log model based on the standard curve of the respective kit lot and the calibrator value (CAL). All essential information on the standard curve is provided on the QC data sheet of the respective product lot.

The calibration curve can be expressed either by the concentration of each standard with its corresponding optical density or by the four parameters A,B,C and D. In both cases the optical density of the calibrator (CAL) is essential.

Depending on your evaluation software program, either the one or the other kind of data described above should be entered.

Caution: Please make sure that all parameters and values are transferred accurately into your software as minor deviations can cause severe errors during evaluation.

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 results have to be multiplied by the **dilution factor of 2 500** (dilution I x dilution II) to get the actual concentrations.

In case **another dilution factor** has been used, multiply the obtained result by the dilution factor used.

9. LIMITATIONS

Samples with concentrations above the measurement range (see definition below) can be further diluted and re-assayed. Please consider this greater dilution when calculating the results.

Samples with concentrations lower than the measurement range (see definition below) cannot be clearly quantified.

The upper limit of the measurement range can be calculated as:

highest concentration of the calibration curve × sample dilution factor to be used

The lower limit of the measurement range can be calculated as:

LoQ × sample dilution factor to be used

LoQ see chapter "Performance characteristics".

10. QUALITY CONTROL

Immundiagnostik AG 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 µg/g⁵ (mg/kg).
- Samples with a calprotectin concentration < 50 µg/g are regarded as negative.
- Samples with a calprotectin concentration between 50 µg/g and 100 µg/g are regarded as borderline positive. We recommend repeating the measurement at a later time point in order to confirm the result.

- Samples with a calprotectin concentration $>100\mu\text{g/g}$ are regarded as positive.

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

Note: Many confounding factors can cause increased levels of faecal 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^{1,10}.

Reference ranges for faecal 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. 95% confidence interval (95% CI) is indicated in brackets.

Age	Number (%)	Median calprotectin [$\mu\text{g/g}$] (95% CI)
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 apparently 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 [$\mu\text{g/g}$]
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 ($<50\mu\text{g/g}$) can be used for children aged 4–17 years.

11. PERFORMANCE CHARACTERISTICS

The following values have been estimated based on the concentrations of the calibration curve without considering possibly used sample dilution factors.

Accuracy – Precision

Repeatability (Intra-Assay); n = 20

The repeatability was assessed with 2 stool samples under **constant** parameters (same operator, measurement system, day and kit lot).

Sample	Mean value [$\mu\text{g/g}$]	CV [%]
1	89.19	5.6
2	229.05	3.2

Reproducibility (Inter-Assay); n = 25

The reproducibility was assessed with 3 stool samples under **varying** parameters (different operators, measurement systems, days and kit lots).

Sample	Mean value [$\mu\text{g/g}$]	CV [%]
1	59.17	11.60
2	253.34	8.82
3	490.31	9.11

Analytical Sensitivity

Limit of blank, LoB	0.957 ng/ml
Limit of detection, LoD	2.267 ng/ml
Limit of quantitation, LoQ	4.767 ng/ml

The evaluation was performed according to the CLSI guideline EP17-A2. The specified accuracy goal for the LoQ was 15 % CV.

Accuracy – Trueness

The trueness states the closeness of the agreement between the result of a measurement and the true value of the measurand. Therefore, calprotectin spikes with known concentrations were added to 2 different stool samples.

Sample [ng/ml]	Spike [ng/ml]	Expected [ng/ml]	Obtained [ng/ml]	Recovery [%]
21.02	173.21	194.23	213.00	109.66
	63.28	84.30	90.76	107.66
	40.48	61.50	62.66	101.90
	17.47	38.49	41.00	106.52
	10.46	31.48	33.28	105.71
18.23	173.21	191.44	188.73	98.58
	63.28	81.52	83.17	102.02
	40.48	58.71	59.34	101.08
	17.47	35.71	35.60	99.71
	10.46	28.69	29.60	103.16

Linearity

The linearity states the ability of a method to provide results proportional to the concentration of analyte in the test sample within a given range. This was assessed according to CLSI guideline EP6-A by means of a ratio dilution of 4 different stool samples.

For calprotectin in stool, the method has been demonstrated to be linear from 15.1 to 1 726.8 µg/ml, showing a non-linear behaviour of less than $\pm 15\%$ in this interval.

Sample	Dilution	Expected [µg/ml]	Obtained [µg/ml]	Recovery [%]
A	50 %	864.2	715.4	82.8
	60 %	1036.7	880.6	84.9
	70 %	1209.3	1091.4	90.3
	80 %	1381.8	1264.2	91.5
	90 %	1554.3	1524.6	98.1
	100 %	1726.8	1726.8	100.0
B	10 %	80.7	85.5	106.0
	20 %	154.3	149.7	97.0
	30 %	227.9	245.7	107.8
	40 %	301.5	305.6	101.3
	50 %	375.1	365.2	97.4
	60 %	448.8	440.7	98.2
	70 %	522.4	507.5	97.1
	80 %	596.04	571.5	95.9
	90 %	669.6	664.2	99.2
	100 %	743.2	743.2	100.0
C	10 %	28.9	32.5	112.4
	20 %	49.2	58.3	118.4
	30 %	69.6	75.0	107.8
	40 %	89.9	103.2	114.8
	50 %	110.2	125.9	114.3
	60 %	130.6	147.2	112.8
	70 %	150.9	176.4	116.9
	80 %	171.2	185.9	108.6
	90 %	191.5	211.9	110.6
	100 %	211.9	211.9	100.0

Sample	Dilution	Expected [µg/ml]	Obtained [µg/ml]	Recovery [%]
D	10 %	15.1	14.9	99.0
	20 %	22.4	21.1	94.1
	30 %	29.7	33.61	113.2
	40 %	37.1	38.7	104.3
	50 %	44.4	45.3	102.0
	60 %	51.7	52.0	100.5
	70 %	59.0	57.7	97.7
	80 %	66.4	64.8	97.6
	90 %	73.7	77.5	105.2
	100 %	81.0	81.0	100.0

Traceability

The calibrator of the IDK® Calprotectin ELISA is based on recombinant human calprotectin which has been calibrated against purified MRP8/14 derived from human granulocytes.

Analytical specificity

The specificity of the antibody was tested by measuring the cross-reactivity against a range of compounds with structural similarity to calprotectin. There was no cross-reactivity observed.

Substance tested	Concentration added [ng/ml]	Concentration obtained [ng/ml]	Conclusion
Lysozyme	10 000	< 0.957	< LoB
PMN Elastase	1 000	2.003	0.2 %
MPO	10 000	< 0.957	< LoB
Laktoferrin	100 000	< 0.957	< LoB

12. PRECAUTIONS

- All reagents in the kit package are for *in vitro* diagnostic use only.
- Control samples should be analysed 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 colour reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
- The 10x Wash buffer concentrate (WASHBUF) contains surfactants which may cause severe eye irritation in case of eye contact.

Warning: Causes serious eye irritation. **IF IN EYES:** Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: get medical Advice/attention.

- 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.
- Do not mix plugs and caps from different reagents.
- The assay should always be performed according to 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.
- IDK® and IDK Extract® are trademarks of Immundiagnostik AG.
- 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 sent to Immundiagnostik AG along with a written complaint.

15. REFERENCES

General literature

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Used symbols:

	Temperature limitation		Catalogue number
	In Vitro Diagnostic Medical Device		To be used with
	Manufacturer		Contains sufficient for <n> tests
	Lot number		Use by
	Attention		Consult instructions for use
	Consult specification data sheet		Irritant