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**Manual**

# *IDK*® Calprotectin ELISA

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

Valid from 2019-01-01

REF K 6928

Σ 96

+8 °C
+2 °C

IVD

CE

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## 1. INTENDED USE

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

## 2. INTRODUCTION

**Alternative names of calprotectin:**

- MRP8/14, L1, (p8,14), p34, S100 A8/A9

**Alternative names of the two proteins forming the heterocomplex calprotectin:**

- S100A8, calgranulin A, MRP8 (migration inhibition factor-related protein-8), CP-10 (in mouse)
- S100A9, calgranulin B, MRP14 (migration inhibition factor-related protein-14)

Calprotectin is a heterodimeric complex, which consists of the calcium-binding proteins S100A8 and S100A9. It is expressed in a tissue-specific manner mainly in cells of the myeloid lineage, including monocytes, neutrophils and earlydifferentiation states of macrophages. However, expression of calprotectin is also inducible in mature macrophages, osteoclasts, keratinocytes, fibroblasts and microvascular endothelial cells (Foell, Roth, 2004).

Calprotectin is involved in innate immunity, leukocyte adhesion, and endothelial transmigration. In addition, it also emerges as important mediator of diverse processes within chronic inflammation, with recent attention being focused on its involvement in inflammation-associated carcinogenesis (Gebhardt et al., 2006).

Indeed, strong up-regulation of the S100A8/A9 proteins has been observed in many tumors, including gastric, colon, pancreatic, bladder, ovarian, thyroid, breast and skin cancers, with both tumor cells and infiltrating immune cells in the tumor stroma being a source of calprotectin (Srikrishna, 2012).

**Indications**

- Detection of urothelial (transitional cell) carcinoma of the bladder

**Exclusion criteria**

- Acute kidney injury, renal transplantation, urinary tract infection, pyuria, previous BCG treatment, secondary transurethral resection of the bladder tumor

## 3. MATERIAL SUPPLIED

| Cat. No. | Label | Kit components | Quantity |
|---|---|---|---|
| K 6928 | PLATE | Holder with precoated strips | 12 x 8 wells |

| Cat. No. | Label | Kit components | Quantity |
|---|---|---|---|
| K 0001.C.100 | WASHBUF | Wash buffer concentrate 10x | 2 x 100 ml |
| K 6928 | SAMPLEBUF | Sample dilution buffer, ready-to-use | 1 x 100 ml |
| K 6928 | STD | Calprotectin standards, ready-to-use (0; 13; 52; 210; 840 ng/ml) | 1 x 5 vials |
| K 6928 | CTRL 1 | Control, ready-to-use (see specification for range) | 1 x 1 vial |
| K 6928 | CTRL 2 | Control, ready-to-use (see specification for range) | 1 x 1 vial |
| K 6928 | CONJ | Conjugate, ready-to-use | 15 ml |
| K 0002.15 | SUB | Substrate (tetramethylbenzidine), ready-to-use | 15 ml |
| K 0003.15 | STOP | Stop solution, ready-to-use | 15 ml |

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

## 4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultra pure water*
- Calibrated precision pipettors and 10–1000 µl single-use tips
- Foil to cover the microtiter plate
- Multi-channel pipets or repeater pipets
- Vortex
- Standard single-use laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader (required filters see chapter 7)

* 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. STORAGE AND PREPARATION OF REAGENTS

- To run the assay more than once, ensure that reagents are stored at the 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.

- **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**.
- 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 storage*

Calprotectin is stable in undiluted urine for 14 months at -20 °C.

### *Sample preparation*

Urine samples should be diluted **1:10 with sample dilution buffer** (SAMPLEBUF) before performing the assay.

## 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.

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 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 sample. A dose response curve of the absorbance unit (optical density, OD at 450 nm) vs. concentration is generated, using the values obtained from the standard. Calprotectin, present in the patient samples, is determined directly from this curve.

## *Test procedure*

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

Mark the positions of standards/controls/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 standards/controls/samples** into the respective wells. |
| 2. | Cover plate tightly 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 plate tightly 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 min** at room temperature (15–30 °C) **in the dark***. |
| 9. | Add **100 µl stop solution** (STOP) into each well, mix thoroughly. |
| 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 observing the colour change and stopping 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.

**Urine**

For calculation of calprotectin concentration in urine, the result must be multiplied by the dilution factor of **10**.

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 standard curve* × *sample dilution factor to be used*

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

*LoB* × *sample dilution factor to be used*

## 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*

- The median value of calprotectin in urine in healthy adults is about 51 ng/ml (interquartile range 20.5–86.6 ng/ml).
- A cut-off of 140 ng/ml has been shown to differentiate best between patients with urothelial bladder carcinoma and healthy subjects (Ebbing et al).

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

## 11. PERFORMANCE CHARACTERISTICS

### *Accuracy – Precision*

**Repeatability (Intra-Assay); n=27**

The repeatability was assessed with 3 urine samples under constant parameters (same operator, measurement system, day and kit lot).

| Sample | Mean value [ng/ml] | CV [%] |
|---|---|---|
| 1 | 4456.5 | 6.2 |
| 2 | 4160.7 | 6.5 |
| 3 | 1238.9 | 5.3 |

**Reproducibility (Inter-Assay); n=11**

The reproducibility was assessed with 4 urine samples under varying parameters (different operators, measurement systems, days and kit lots).

| Sample | Mean value [ng/ml] | CV [%] |
|---|---|---|
| 1 | 2368.5 | 8.1 |
| 2 | 211.2 | 7.7 |
| 3 | 524.4 | 5.8 |
| 4 | 5133.7 | 12.5 |

## *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 urine samples. The results below were obtained without consideration of the sample dilution factor.

| Sample [ng/ml] | Spike [ng/ml] | Expected [ng/ml] | Obtained [ng/ml] | Recovery [%] |
|---|---|---|---|---|
| 18.6 | 233.0 | 251.6 | 294.2 | 116.9% |
| | 116.5 | 135.1 | 155.1 | 114.8% |
| | 58.3 | 76.8 | 84.0 | 109.4% |
| | 29.1 | 47.7 | 47.1 | 98.8% |
| | 14.6 | 33.1 | 30.1 | 90.7% |
| | 7.3 | 25.9 | 23.3 | 90.1% |
| | 3.6 | 22.2 | 20.2 | 91.0% |
| 43.7 | 233.0 | 276.7 | 313.3 | 113.2% |
| | 116.5 | 160.2 | 182.8 | 114.1% |
| | 58.3 | 101.9 | 110.1 | 108.0% |
| | 29.1 | 72.8 | 73.1 | 100.5% |
| | 14.6 | 58.2 | 56.3 | 96.7% |
| | 7.3 | 50.9 | 49.6 | 97.4% |
| | 3.6 | 47.3 | 45.9 | 97.0% |

## *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 with a serial dilution of 2 different urine samples.

For calprotectin urine, the method has been demonstrated to be linear from 10.25 to 508.70 ng/ml based on the standard curve without considering possibly used sample dilution factors, showing a non-linear behaviour of less than ±20 % in this interval.

| Sample | Dilution | Expected [ng/ml] | Obtained [ng/ml] | Recovery [%] |
|---|---|---|---|---|
| A | 1:20 | 508.70 | 508.70 | 100.00 |
| | 1:40 | 254.35 | 285.38 | 112.20 |
| | 1:80 | 127.18 | 154.41 | 121.42 |
| | 1:160 | 63.59 | 73.35 | 115.35 |
| | 1:320 | 31.79 | 35.44 | 111.48 |
| | 1:640 | 15.90 | 17.51 | 110.13 |
| B | 1:10 | 433.46 | 433.46 | 100.00 |
| | 1:20 | 216.73 | 218.10 | 100.63 |
| | 1:40 | 108.36 | 104.89 | 96.80 |
| | 1:80 | 54.18 | 48.91 | 90.28 |
| | 1:160 | 27.09 | 22.38 | 82.60 |
| | 1:320 | 13.55 | 10.25 | 75.71 |

## *Analytical sensitivity*

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

Limit of blank, LoB 0.985 ng/ml

Limit of detection, LoD 1.509 ng/ml

Limit of quantitation, LoQ 1.509 ng/ml

The evaluation was performed according to the CLSI guideline EP17-A2. The specified accuracy goal for the LoQ was 20 % CV.

## *Traceability*

The standards of the IDK® Calprotectin ELISA are 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 | < 0.985 | < LoB |
| PMN Elastase | 1 000 | 2.003 | 0.2 % |
| MPO | 10 000 | < 0.985 | < LoB |
| Laktoferrin | 100 000 | < 0.985 | < LoB |

## 12. PRECAUTIONS

- All reagents in the kit package are for *in vitro* diagnostic use only.
- 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 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 up 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 assembling wells of different microtiter plates for analysis, even if they are of the same batch.
- Control samples should be analysed with each run.
- 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.
- The guidelines for medical laboratories should be followed.
- *IDK*® is a trademark 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 incorrect use.
- Warranty claims and complaints regarding 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. Foell, Dirk, and Johannes Roth. 2004. "Proinflammatory S100 Proteins in Arthritis and Autoimmune Disease." *Arthritis and Rheumatism* **50** (12) (December): 3762–71. doi:10.1002/art.20631.
2. Gebhardt, Christoffer, Julia Németh, Peter Angel, and Jochen Hess. 2006. "S100A8 and S100A9 in Inflammation and Cancer." *Biochemical Pharmacology* **72** (11) (November 30): 1622–31. doi:10.1016/j.bcp.2006.05.017.
3. Srikrishna, Geetha. 2012. "S100A8 and S100A9: New Insights into Their Roles in Malignancy." *Journal of Innate Immunity* **4** (1) (January): 31–40. doi:10.1159/000330095.

## *Literature using Immundiagnostik Calprotectin ELISA*

4. Ebbing, Jan, Susanne Mathia, Felix S Seibert, Nikolaos Pagonas, Frederic Bauer, Barbara Erber, Karsten Günzel, et al. 2014. "Urinary Calprotectin: A New Diagnostic Marker in Urothelial Carcinoma of the Bladder." *World Journal of Urology* **32** (6): 1485–92. doi:10.1007/s00345-013-1227-8.

***Used symbols:***