PhiCal® Calprotectin ELISA Kit

Zur in-vitro-Bestimmung von Calprotectin (MRP 8/14, S100A8/A9) in Serum, Plasma und Urin

PhiCal® Calprotectin ELISA Kit

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

[PhiCal®: registered German trademark of Immundiagnostik AG]

Gültig ab / Valid from 27.08.2013

REF  K 6935  \( \sum \)

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Immundiagnostik AG
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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 of calprotectin:**
- MRP8/14, L1, (p8,14), p34

**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 calcium-binding protein secreted predominantly by neutrophils and monocytes. The heterocomplex consists of the two proteins, S100A8 (calgranulin A) and S100A9 (calgranulin B), also designated as MRP8 and MRP14, respectively. Expression of S100A8 and S100A9 in epithelial tissues was first described in context with squamous epithelia and with murine and human wound repair. More recently, an association of S100 protein expression with adenocarcinomas in humans has emerged. The genes S100A8 and S100A9 are located in a gene cluster on chromosome 1q21, a region in which several rearrangements that occur during tumor development have been observed.

Elevated MRP8/14 levels have been found in many sites of inflammation and in the extracellular fluid of patients with many types of inflammatory conditions. The concentration of MRP8/14 in blood is increased in patients with rheumatoid arthritis, cystic fibrosis, multiple sclerosis, and HIV infections, while elevated MRP8/14 levels have been detected in stool of patients with Crohn’s disease and colorectal cancer [1-5]. Extracellular MRP8/14 has antimicrobial, antigrowth and apoptotic effects. It suppresses the growth of some fungi and bacteria [1,2]. It also suppresses the proliferation of several different types of cells including: macrophages, lymphocytes, hematopoietic progenitors, and tumor cell lines. MRP8/14 can also induce apoptosis of some tumor cell lines [1,2].

Hermani et al. (2005) [6] reported recently that enhanced expression of S100A8 and S100A9 is an early event in prostate tumor genesis and may contribute to development and progression or extension of prostate carcinomas. Furthermore, they tested the value of S100A9 as a serum marker for prostate cancer comparing the serum concentrations of S100A9 in cancer patients with healthy controls or patients with benign prostatic hyperplasia (BPH). Significantly increased S100A9 serum levels in prostate cancer were found in prostate cancer patients compared to patients with
BPH, the latter exhibiting values similar to that obtained for healthy individuals.

**Pathological significance and clinical application**

The diagnostic value and advantage of MRP8/14 over other disease markers is that they are preformed and released immediately upon activation of the respective cell population. Other markers may be generated in downstream events or need to be synthesized de novo in the liver. Various conditions have shown significant correlation of MRP8/14 (or MRP8, MRP14) levels with disease activity:

- Concentrations of MRP8/14 in serum, and particularly in synovial fluid, correlate strongly with disease activity in rheumatoid arthritis.
- Plasma MRP8/14 levels are very early, specific and sensitive prediction markers for acute rejection in kidney allograft transplantation.
- Serum MRP8/14 concentration is a prognostic marker of recurrent infection and survival in alcoholic liver cirrhosis.
- MRP8/14 is useful for evaluating the extent of periodontal inflammation.
- In cerebral malaria, MRP 8/14 expression correlates with microglial activation in brain.
- MRP8/14 is present in urinary stones and in dental calculus.
- S100A9 in serum may serve as a useful marker for discrimination between prostate cancer and benign prostatic hyperplasia (BPH).

### 3. MATERIAL SUPPLIED

<table>
<thead>
<tr>
<th>Cat. No.</th>
<th>Label</th>
<th>Kit components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>K 6935MTP</td>
<td>PLATE</td>
<td>Holder with precoated strips</td>
<td>12 x 8 wells</td>
</tr>
<tr>
<td>K 6935WP</td>
<td>WASHBUF</td>
<td>ELISA wash buffer concentrate 10x</td>
<td>2 x 100 ml</td>
</tr>
<tr>
<td>K 6935A2</td>
<td>AB</td>
<td>Detection antibody, (monoclonal anti-Calprotectin (MRP 8/14) antibody, biotinylated), concentrate</td>
<td>50 µl</td>
</tr>
<tr>
<td>K 6935ST</td>
<td>STD</td>
<td>Calprotectin standards, lyophilized (0; 3.9; 15.6; 62.5; 250 ng/ml)</td>
<td>2 x 5 vials</td>
</tr>
<tr>
<td>K 6935KO1</td>
<td>CTRL</td>
<td>Control, lyophilized (see specification for range)</td>
<td>2 x 1 vial</td>
</tr>
<tr>
<td>K 6935KO2</td>
<td>CTRL</td>
<td>Control, lyophilized (see specification for range)</td>
<td>2 x 1 vial</td>
</tr>
<tr>
<td>K 6935K</td>
<td>CONJ</td>
<td>Conjugate, (extravidin peroxidase labeled), concentrate</td>
<td>50 µl</td>
</tr>
</tbody>
</table>
### 4. MATERIAL REQUIRED BUT NOT SUPPLIED

- Ultra pure water*
- Laboratory balance
- Precision pipettors calibrated and tips to deliver 10-1000 µl
- Foil to cover the microtiter plate
- Horizontal microtiter plate shaker with 37 °C incubator
- Multi-channel dispenser or repeating dispenser
- Centrifuge, 3000 g
- Vortex mixer
- 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 WASHBUF + 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** can be stored in a closed flask at **2-8 °C for one month.**

- The **lyophilized STD** (standards) and **CTRL** (controls) are stable at **2-8 °C** until the expiry date stated on the label. Before use, 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 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 **detection antibody** (AB) 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 could not be stored.**

- The **conjugate** (CONJ) 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 cannot be stored.**

- 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

**Preanalytic handling**

Significant differences in the calprotectin levels can be observed due to different sample preparation procedures, e.g. up to 10-fold higher serum levels compared to the plasma calprotectin concentrations. The reasons are as follows:

Granulocytes are activated during serum clotting and release granulocyte-activating markers. The time between serum collecting and analysis as well as repeated freeze-thaw cycles don’t cause a calprotectin concentration shift.

On the contrary, in the case of plasma samples, varying the time between sampling and analysis or the number of freeze-thaw cycles will cause variation in the observed calprotectin levels. Therefore, the preanalytical conditions of plasma samples should be held constant. This is a general requirement independent of the used test-system. Immundiagnostik recommends the use of serum samples for calprotectin determinations.

**Lipemic or hemolytic samples may give erroneous results and should not be used for analysis.**

**Serum samples**

- Serum samples should be diluted **1:50 with wash buffer** before performing the assay.
Plasma samples
EDTA plasma samples should be diluted **1:10 with wash buffer** before performing the assay.

Urine samples
Urine samples should be diluted **1:10 with wash buffer** before performing the assay.

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. 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. Tetramethyl-benzidine (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**
Wash the pre-coated microtiter plate **5 x with 250 µl ELISA wash buffer before use.** After the final washing step, the inverted microtiter plate should be tapped on absorbent paper.

Carry out the tests in duplicate.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Bring all <strong>reagents and samples</strong> to <strong>room temperature</strong> (15 - 30°C) and mix well</td>
</tr>
<tr>
<td>2.</td>
<td>Mark the positions of <strong>STD / SAMPLE / CTRL</strong> (standards / samples / controls) in duplicate on a <strong>protocol sheet</strong></td>
</tr>
</tbody>
</table>
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** (standard / sample / controls) in duplicate into respective well.

5. Cover plate tightly and incubate for **1 hour at 37 °C on a horizontal mixer**

6. Aspirate the contents of each well. Wash 5 times by dispensing **250 µl of wash buffer** into each well. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.

7. Add **100 µl AB** (detection antibody) into each well.

8. Cover plate tightly and incubate for **1 hour at 37 °C on a horizontal mixer**

9. Aspirate the contents of each well. Wash 5 times by dispensing **250 µl of wash buffer** into each well. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.

10. Add **100 µl CONJ** (conjugate) into each well.

11. Cover plate tightly and incubate for **1 hour at 37 °C on a horizontal mixer**

12. Aspirate the contents of each well. Wash 5 times by dispensing **250 µl of wash buffer** into each well. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.

13. Add **100 µl of SUB** (substrate) into each well.

14. Incubate for **10 - 20 minutes at room temperature (15 - 30 °C) in the dark**

15. Add **100 µl of STOP** (stop solution) into each well, mix thoroughly.

16. 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.*
**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.**

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.

**Serum**
For calculation of calprotectin concentration in serum, the result must be multiplied by the dilution factor of **50**.

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

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

9. LIMITATIONS

Samples with an OD greater than the OD of the highest calibrator should be further diluted 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.

Normal range
Calprotectin in serum of healthy persons: < 3 µg/ml (< 3000 ng/ml)
We recommend each laboratory to establish its own norm concentration range.

11. PERFORMANCE CHARACTERISTICS

Precision and reproducibility

Serum samples

*Intra-Assay (n = 7)*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Calprotectin [ng/ml]</th>
<th>CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>878,58</td>
<td>6,16</td>
</tr>
<tr>
<td>2</td>
<td>1750,10</td>
<td>4,48</td>
</tr>
<tr>
<td>3</td>
<td>877,13</td>
<td>4,90</td>
</tr>
</tbody>
</table>

*Inter-Assay (n = 13)*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Calprotectin [ng/ml]</th>
<th>CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>768,62</td>
<td>7,49</td>
</tr>
<tr>
<td>2</td>
<td>813,97</td>
<td>10,32</td>
</tr>
<tr>
<td>3</td>
<td>1584,67</td>
<td>12,86</td>
</tr>
</tbody>
</table>
Plasma samples

**Intra-Assay (n = 7)**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Calprotectin [ng/ml]</th>
<th>CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>269,92</td>
<td>5,28</td>
</tr>
<tr>
<td>2</td>
<td>276,35</td>
<td>4,83</td>
</tr>
<tr>
<td>3</td>
<td>213,69</td>
<td>4,43</td>
</tr>
</tbody>
</table>

**Inter-Assay (n = 12)**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Calprotectin [ng/ml]</th>
<th>CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>201,26</td>
<td>13,23</td>
</tr>
<tr>
<td>2</td>
<td>298,37</td>
<td>12,76</td>
</tr>
<tr>
<td>3</td>
<td>184,97</td>
<td>13,06</td>
</tr>
</tbody>
</table>

**Spiking Recovery**

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

**Serum samples**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Unspiked Sample [ng/ml]</th>
<th>Spike [ng/ml]</th>
<th>Calprotectin expected [ng/ml]</th>
<th>Calprotectin measured [ng/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>24,1</td>
<td>20</td>
<td>44,1</td>
<td>41,3</td>
</tr>
<tr>
<td></td>
<td>24,1</td>
<td>30</td>
<td>54,1</td>
<td>50,9</td>
</tr>
<tr>
<td>B</td>
<td>16,5</td>
<td>20</td>
<td>36,5</td>
<td>34,1</td>
</tr>
<tr>
<td></td>
<td>16,5</td>
<td>30</td>
<td>46,5</td>
<td>46,1</td>
</tr>
<tr>
<td></td>
<td>16,5</td>
<td>40</td>
<td>56,5</td>
<td>53,8</td>
</tr>
</tbody>
</table>
Plasma samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Unspiked Sample [ng/ml]</th>
<th>Spike [ng/ml]</th>
<th>Calprotectin expected [ng/ml]</th>
<th>Calprotectin measured [ng/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18,9</td>
<td>20</td>
<td>38,9</td>
<td>36,3</td>
</tr>
<tr>
<td></td>
<td>18,9</td>
<td>40</td>
<td>58,9</td>
<td>55,8</td>
</tr>
<tr>
<td></td>
<td>18,9</td>
<td>60</td>
<td>78,9</td>
<td>74,3</td>
</tr>
<tr>
<td>B</td>
<td>17,1</td>
<td>40</td>
<td>57,1</td>
<td>60,6</td>
</tr>
<tr>
<td></td>
<td>17,1</td>
<td>60</td>
<td>77,1</td>
<td>74,9</td>
</tr>
<tr>
<td></td>
<td>17,1</td>
<td>80</td>
<td>97,1</td>
<td>94,8</td>
</tr>
</tbody>
</table>

Dilution recovery

Two patient samples were diluted and analyzed. The results are shown below (n = 2):

Serum samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Calprotectin expected [ng/ml]</th>
<th>Calprotectin measured [ng/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1:50</td>
<td>1425,38</td>
<td>1425,38</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>712,69</td>
<td>744,54</td>
</tr>
<tr>
<td></td>
<td>1:200</td>
<td>356,34</td>
<td>355,33</td>
</tr>
<tr>
<td>B</td>
<td>1:50</td>
<td>1026,11</td>
<td>1026,11</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>513,05</td>
<td>502,04</td>
</tr>
<tr>
<td></td>
<td>1:200</td>
<td>256,52</td>
<td>256,91</td>
</tr>
</tbody>
</table>

Plasma samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Calprotectin expected [ng/ml]</th>
<th>Calprotectin measured [ng/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1:10</td>
<td>244,29</td>
<td>244,29</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>122,14</td>
<td>115,10</td>
</tr>
<tr>
<td></td>
<td>1:40</td>
<td>61,10</td>
<td>57,67</td>
</tr>
</tbody>
</table>
## Analytical Sensitivity

The Zero-standard was measured 20 times. The detection limit (limit of blank, LoB) was set as $B_0 + 2 \text{ SD}$ and estimated to be 3,2 ng/l.

## Specificity

No cross-reactivity with MPR 8/14 in mouse serum was observed.

No cross-reactivity was observed to the following plasma proteins:

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

### 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


2. Yui, S., Nakatani, Y. & Mikami, M. Calprotectin (S100A8/S100A9), an inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. Bio-


**Literature using Immundiagnostik Calprotectin ELISA**


Used symbols:

- **Temperature limitation**
- **Catalogue Number**
- **In Vitro Diagnostic Medical Device**
- **Contains sufficient for \(<n>\) tests**
- **Manufacturer**
- **Use by**
- **Lot number**