MRP8 (S100A8)
Enzyme Immunoassay

for the specific determination of MRP8 in biological fluids
Test Instructions

Product Code: S-1007
Lot number: 23E1203

For *in vitro* and research use only.
Not for diagnostic applications.

Kit contains: 2 precoated, dry microtiter plates and reagents to perform 2x96 tests.
Refrigerate upon arrival, do not freeze.

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Introduction and Basic Information

Alternative names:
MRP8: S100A8, Calgranulin A, CP-10 (in mouse)
MRP14: S100A9, Calgranulin B
MRP8/14: Calprotectin, L1, (p8,14), p34

Migration inhibitory factor-related proteins (MRP) -8 and -14 belong to the S-100 family of calcium binding proteins associated with myeloid cell differentiation. They are highly expressed in resting neutrophils, keratinocytes (particularly in psoriasis), in infiltrating tissue macrophages and on epithelial cells in active inflammatory disease. The heterogeneity of macrophage subpopulations in chronic or acute inflammation is reflected by different expression of MRP8 and MRP14. Phagocytes expressing MRP8 and MRP14 belong to the early infiltrating cells, while MRP8 alone is found in chronic inflammatory tissues. The partially antagonistic functions of MRP8, MRP14 and of the Ca^{2+}-dependent MRP8/14 heterocomplex makes them versatile mediators.

Functions

One major function of the MRP8/14 heterocomplex is its antimicrobial activity (hence the name calprotectin). MRP8/14 inhibits the growth of pathogens through competition for zinc. MRP8/14 also induces apoptosis of certain tumor cells. These activities are abrogated by Zn^{2+} and other divalent cations, but not by Ca^{2+} or Mg^{2+}.

Another important property specific for the MRP8/14 heterocomplex is its unique role as a fatty acid transport protein. The Ca^{2+}-dependent fatty acid-MRP8/14 complex is the major carrier of polyunsaturated fatty acids in neutrophils. The complex is expressed in resting cells and moves to the membrane upon stimulation. Zn^{2+} inhibits the fatty acid carrier capacity of MRP8/14 already at physiological Zn^{2+} serum concentrations, so that fatty acids are not carried in the blood circulation. This makes MRP8/14 an important mediator between calcium signaling and arachidonic acid effects.

MRP8 (and MRP8/14, but not MRP14 alone) is secreted upon stimulation with inflammatory mediators. It is a potent chemoattractant for neutrophils and monocytes. However, MRP8 does not increase intracellular Ca^{2+} nor evoke an oxidative burst and granular enzyme release like e.g. C5a. Exposure of MRP8 to hypochlorite, possibly generated by activated neutrophils, converts it to the inactive disulfide-linked dimer. Glucocorticoids up-regulate induction of MRP8 by inflammatory mediators. MRP8 may contribute to the regulation of fetal-maternal interactions, which would explain why inactivation of the MRP8 gene in the mouse is embryonic lethal.

The lack of co-expression of MRP14 with MRP8 in activated macrophages points to their different roles. The C-terminal sequence of MRP14 is identical to the N-terminus of neutrophil immobilizing factors. MRP14 can be phosphorylated which increases its Ca^{2+}-binding capacity. It then tends to move from the cytosol to membranes and the cytoskeleton. MRP14 has been shown to be associated with a subpopulation of neutrophils with metastasis-enhancing abilities.

Biochemistry

Human MRP8 has a molecular weight of 11.0kD, while human MRP14 exists in a 13.3kD and a truncated 12.9kD form. Ca^{2+} induces the formation of heterocomplexes of the form (MRP8)(MRP14) (abbreviated MRP8/14), $(MRP8)_2(MRP14)$, and $(MRP8/14)_2$. There are two EF-hand motifs each on MRP8 and MRP14. MRP14 shows a higher affinity for calcium than MRP8, and the affinity of the C-terminal EF2 is higher than that of the N-terminal EF1. The C-terminal domain also mainly determines the specificity of dimerization. The helix in EF2 undergoes a large conformational change upon calcium binding and may play a role as a trigger for Ca^{2+} induced conformational change.

The antimicrobial activity of MRP8/14 is caused by zinc chelation by a polyhistidine sequence near the C-terminus of MRP14 and is reversed by Zn^{2+}. Neither one of the subunits shows antibacterial activity by itself, indicating that Ca^{2+} induced dimerization leads to an altered exposure of the polyhistidine sequence.

The Ca^{2+}-induced binding of arachidonic and polyunsaturated fatty acids to MRP8/14 is prevented by addition of Zn^{2+} or Cu^{2+} by affecting the conformation of the calcium-dependent fatty acid binding pocket. Maximal fatty acid binding occurs at equimolar concentrations of MRP8 and MRP14 and for values greater than 3 calcium ions per EF-hand.

Pathological significance

MRP8/14 and MRP14 are generally associated with acute, and MRP8 with chronic inflammatory conditions. The diagnostic value and advantage of MRPs 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 the MRP8/14 heterocomplex levels with disease activity. However, changes in the level of the MRP8 subunit have received little attention.

- Fecal MRP8/14 levels predict relapse in inflammatory bowel diseases and distinguish between healthy controls, patients with no or low disease activity and patients with active disease.
- Plasma MRP8/14 levels are possible markers for acute rejection in kidney allograft transplantation.
- Serum MRP8/14 concentration is a prognostic marker of recurrent infection and of poor survival in alcoholic liver cirrhosis.
- Concentrations of MRP8/14 in serum, and particularly in synovial fluid, correlate strongly with disease activity in rheumatoid arthritis. In SLE patients, serum levels of MRP8/14 are higher than in healthy controls and are associated with disease activity, with the presence of anti DNA antibodies, and with the occurrence of arthritis.
- MRP8 and MRP14 can be detected in age-related cerebral changes and neurodegenarative disorders. In cerebral malaria, microglial activation and detection of MRP8/14 is widespread throughout the brain.
- MRP8 (originally also called cystic fibrosis (CF) antigen) is a superior index of inflammation in CF. It is constitutively expressed in the lungs and serum of CF patients and is elevated in the plasma of patients who are not acutely unwell or pyrexic. LPS seems to induce MRP8 in CF to a greater extent than in normals.
- MRP8/14 is present in urinary stones and in dental calculus. The MRP8/14 level in gingival crevicular fluid correlates well with other markers of periodontal disease and makes MRP8/14 useful for evaluating the extent of periodontal inflammation.

MRP patterns have been qualitatively reported as follows:

	MRP8	MRP14	MRP8/14
normal	Low	Low	Low (<3500ng/ml)
acute inflammation	Low	Variable	High
chronic inflammation	Elevated	High	Low
acute phase within chronic inflammation	Elevated	High	High

Still, normal levels of MRP8 or MRP14 are at least three orders of magnitude below MRP8/14 levels.

Test principle

One-step non-competitive sandwich assay, reagent limited with peroxidase catalyzed Tetramethylbenzidine color reaction, including a stop reaction and reading at 450/630nm in a multititer plate reader.

Reagents provided

S-1007A:	Ready-to-use precoated and stabilized microtiter plate + plate sealer (2 each).
S-1007B	500ng standard, a stabilized lyophilized preparation of recombinant MRP8.
S-1007D	Assay buffer, 3x concentrated. Red solution.
S-1007E	Tetramethylbenzidin – H_2O_2 solution. Keep in the dark.
S-1007F	TMB substrate buffer (potassium citrate)
S-1007R	Reagent mixture: 2.1ml of a mixture including an HRPO-coupled detection antibody, 11x concentrated

The kit is stable at least until the date indicated when stored refrigerated.

Material not provided: STOP solution (1N sulfuric acid, see below), plastic tubes for dilutions; isotonic saline for washing, pipettes, repeater pipette, 8-channel pipettes (2), microplate washer and reader (450nm/630nm filters).

Preparations before you start the assay

Let all the reagents warm up to room temperature before starting. Duplicate testing of samples and standards is highly recommended. Prepare all of the following dilutions *except for the TMB Substrate Solution* before you start the assay:

Assay Buffer

Dilute 1 part of the enclosed concentrate (S-1007D) with 2 parts distilled water to obtain the Assay Buffer. Example: measure 15ml of concentrate and add 30ml water.

Standards:

Reconstitute lyophilized standard with 1.0ml Assay Buffer. Vortex thoroughly several times for 10 seconds with a few minutes incubation in between. This 500ng/ml standard is now ready for further dilution. Store unused portion of this standard at -20°C for further use, if necessary.

Dilutions:
- Label five 1.5ml polypropylene tubes with "25", "5", "1", "0.2" and "0.04".
- Add 950µl assay buffer to the "25" tube, 400µl assay buffer to the four others
- Make a serial dilution by adding 50µl of the 500ng/ml stock solution to the "25" tube. Mix well, transfer 100µl of this solution to the "5" tube and continue a serial dilution by always transferring 100µl to the next tube in decreasing order. This gives five standard solutions for generating a standard curve with duplicate standards.

Reagent Mixture (working dilution)

Dilute 1 part of the enclosed Reagent Mixture (S-1007R) with 10 parts Assay Buffer. Example: add 0.32ml Reagent Mixture to 3.2ml Assay Buffer if you process four strips containing 32 wells (each well will receive 100µl). Adjust this volume according to your needs.

Samples:

Store samples in aliquots at –20°C or lower. Try to avoid freeze-thaw cycles with your samples and dilute your sample in appropriately diluted Assay Buffer. Serum or plasma should be diluted 1:5. Example: Spin your sample in a tabletop centrifuge for 5 minutes, then add 100µl to 400µl Assay Buffer. Use 1:20 - 1:1000 for synovial fluid; 1:2 for exhalates, saliva, BAL and urine.

Isotonic saline:

Dissolve 9g of NaCl in 1 liter water, deionized or similar quality.

TMB Substrate Solution:

Prepare immediately before use. For one whole plate, mix 20ml substrate buffer (S-1007F) with 1ml substrate stock solution (S-1007E). Use within 15 minutes after preparation.

STOP solution
Dilute sulfuric acid to a concentration of **1N**. Example: Add 2.9ml 95-97% sulfuric acid to 100ml water (in this sequence). 95-97% concentrated sulfuric acid (specific gravity 1.84) is 36N. This solution is best prepared before starting the assay.

Test procedure

1. Add 100µl diluted Reagent Mixture (see above) to each well. A repeater pipette dispensing 100µl is most convenient if you work several columns.

2a. Add 100µl each of the 25ng/ml ("25") to 0.04ng/ml ("0.04") standard to wells down columns 1 and 2 as suggested below. Include two blanks (if working with duplicate samples) which get 100µl Assay Buffer.

2b. Add 100µl of appropriately diluted sample to the corresponding wells.

3. Incubate at room temperature for 90 minutes in a humid environment.

4. Wash the plate 3 times with isotonic saline. Blot onto a soft absorbing paper.

Dilute TMB Substrate Solution at this time, have STOP solution and 8-channel pipettes ready to stop color reaction in time.

5. Add 200µl diluted TMB substrate solution to each well. Incubate for ***3 minutes*** at room temperature. A blue color reaction occurs where MRP8 is present.

6. Stop color reaction by adding 100µl stop solution to each well. Coloration turns from blue to yellow. **Caution:** Stop solution contains 1N sulfuric acid which is corrosive and causes burns. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

7. Within about 15 minutes, read absorbance at 450nm with reference set to 630nm, if possible.

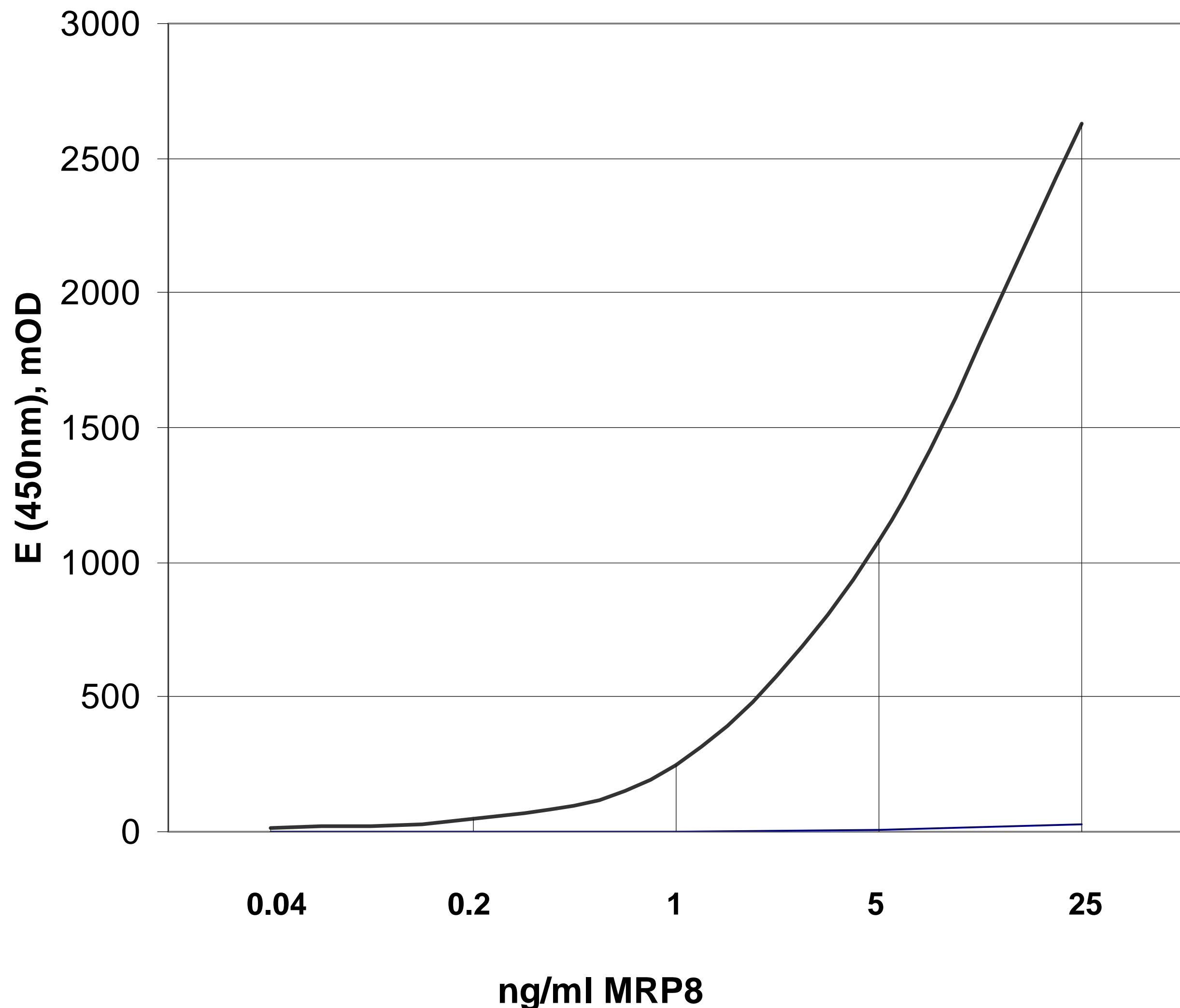
Suggested plate set-up:

The following plate arrangement may be chosen, where 100 through 0.39 are the standard dilutions from 100ng/ml to 0.39ng/ml, as described above. Sa-1 through Sa-40 are samples in duplicates. Cont refers to a control serum with a known MRP8 content (not included in the kit).

	1	2	3	4	5	6	7	8	9	10	11	12
A	25	25	Sa-3	Sa-3	Cont	Cont	Sa-18	Sa-18	Sa-26	Sa-26	Sa-34	Sa-34
B	5	5	Sa-4	Sa-4	Sa-11	Sa-11	Sa-19	Sa-19	Sa-27	Sa-27	Sa-35	Sa-35
C	1	1	Sa-5	Sa-5	Sa-12	Sa-12	Sa-20	Sa-20	Sa-28	Sa-28	Sa-36	Sa-36
D	0.2	0.2	Sa-6	Sa-6	Sa-13	Sa-13	Sa-21	Sa-21	Sa-29	Sa-29	Sa-37	Sa-37
E	0.04	0.04	Sa-7	Sa-7	Sa-14	Sa-14	Sa-22	Sa-22	Sa-30	Sa-30	Sa-38	Sa-38
F	Blank	Blank	Sa-8	Sa-8	Sa-15	Sa-15	Sa-23	Sa-23	Sa-31	Sa-31	Sa-39	Sa-39
G	Sa-1	Sa-1	Sa-9	Sa-9	Sa-16	Sa-16	Sa-24	Sa-24	Sa-32	Sa-32	Sa-40	Sa-40
H	Sa-2	Sa-2	Sa-10	Sa-10	Sa-17	Sa-17	Sa-25	Sa-25	Sa-33	Sa-33	Cont	Cont

Calculation

Means are formed from duplicates and the content in the samples is calculated from the standard curve with the help of a microplate calculation software (e.g. Softmax, Molecular Device) or manually. Sample dilutions which lie outside of the standard range should be repeated with the appropriate dilution.



Limitations and incompatibilities

Components from different lots or from different assays should not be mixed.

Serum and plasma samples can give comparable results. However, blood samples need to be chilled immediately after drawing and processed to plasma or serum, respectively, right away.

Room temperature and incubation time affect all reactions more or less. If it happens that some values are off scale (E^{450nm} >3.0) we suggest to remove the same volume of solution from every well (e.g. 100µl) and re-measure the plate.

Interpretation of the Results

The circulating levels of MRP8/14 are a good indicator of pathological conditions. Additionally, the levels of the circulating subunits MRP8 and MRP14 can be measured as well and may give interesting clues to the pathogenesis of a disease. The normal range for MRP subfamilies measured in serum or plasma is:

MRP8/14	500 – 3000ng/mL
MRP8	< 2ng/mL
MRP14	< 2ng/mL

The concentration of the MRP8/14 complex is an indication of the severity of inflammation (extreme values >100,000 ng/mL have been measured in serum and plasma. C-reactive protein (CRP) and other inflammation markers do not always correlate with MRP8/14 because MRP levels increase earlier than those of acute phase proteins like CRP.

Subnormal levels of MRP8/14 (<100 ng/mL) may indicate a disturbance of granulocyte differentiation.

An elevated MRP8 concentration indicates chronic inflammation. It has been shown that 93% of patients with rheumatoid arthritis show elevated MRP8 values. However, MRP8 levels are within normal range in cases of acute inflammation such as activated arthritis and bacterial infections.

Acute inflammation, such as bacterial infection is characterized by:

- Normal MRP8 concentration
- Normal to elevated MRP14 concentration
- High concentration of the MRP8/14 complex (>3,000-100,000 ng/mL serum)

Chronic inflammation, such as rheumatoid arthritis is characterized by:

- A high MRP8 concentration
- A high MRP14 concentration
- Slightly elevated concentrations of the MRP8/14 heterocomplex. The MRP8/14 concentration is clearly elevated in an acute phase of chronic inflammation.

Viral infection alone does not result in elevated MRP8/14 concentration. Sera of pancreatitis patients do not show elevated MRP8/14 serum concentrations.

Immunesuppressive treatment with glucocorticoids causes MRP8/14 heterocomplex levels to return to normal range

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