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MRP14

Enzyme Immunoassay

Test for the determination of MRP8 in biological fluids

Test Instructions

Product Code: S-1008

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), $(\text{MRP8})_2(\text{MRP14})$, and $(\text{MRP8/14})_2$. 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 MRP8/14 (or MRP8) levels with disease activity:

- 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 of poor survival in alcoholic liver cirrhosis.
- 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.
- 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 neurodegenerative 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 pyrexia. 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.

Typical MRP patterns can be summarized as follows:

	MRP8	MRP14	MRP8/14
normal	Low (5-15ng/ml)	Low (<5ng/ml)	Low (<3500ng/ml)
acute inflammation	Low (5-15ng/ml)	Variable	High
chronic inflammation	High	High	Low
acute phase within chronic inflammation	High	High	High

Test principle

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

The test has been adjusted in a way to minimize cross reactivity with the MRP8/14 complex.

Reagents provided

S-1008A:	Ready-to-use precoated and stabilized microtiter plate (Nunc Maxisorb) + 1 plate sealer (2 of each per kit).
S-1008B	500ng standard, a stabilized lyophilized preparation of recombinant MRP14.
S-1008C	15µg biotinylated detection antibody, lyophilized. 1 vial per kit.
S-1008D	Assay buffer (3x concentrated) Slightly turbid solution.
S-1008E	Tetramethylbenzidine (TMB) – H ₂ O ₂ solution. Keep dark.
S-1008F	Substrate buffer (citrate).
S-1008H	60µl Streptavidin-Peroxidase conjugate. Spin briefly to retrieve all the solution.
S-1008J	Blocking reagent, concentrated; 1 vial containing 2.1ml.

The kit is stable for 6 months after delivery when stored refrigerated in original packaging.

Material not provided: STOP solution (1N sulfuric acid, see below), plastic tubes for standard dilution; pipettes and microplate reader with 450nm filter.

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 the following dilutions immediately before use:

Assay Buffer

Dilute 1 part of the enclosed concentrate with 2 parts water to obtain the working dilution of the Assay Buffer.

Blocking Reagent

Dilute 1ml of the enclosed blocking reagent with 10ml Assay Buffer. This is sufficient for 1 plate. Adjust the volumes to your particular needs if not a whole plate is used.

Samples:

Store samples in aliquots at –20°C or lower. Use an aliquot only once. We recommend a dilution of 1:5 for normal serum or plasma. Suggestion: vortex sample briefly after thawing, spin for 5 minutes in a microcentrifuge, then add 100µl sample to 400µl Assay Buffer. Mix gently and measure sample in duplicate. No unwanted reactions have been observed with lipaemic samples, but care had been taken to remove the desired plasma volume from underneath the lipid layer.

For synovial fluid we recommend a dilution of 1:100 - 1:10,000. For exhalates, saliva, bronchoalveolar lavage fluid (BAL) and urine we suggest a dilution of 1:2.

We have noticed that EDTA plasma has a tendency to coagulate after dilution with assay buffer and incubation at 37°C. This kit includes a newly formulated assay buffer where this should not happen easily anymore.

Standards:

Reconstitute lyophilized standard with 1.0ml assay buffer to obtain a 500ng/ml stock solution. Vortex gently, incubate for 10 minutes at room temperature and vortex gently again. Dilute this stock solution 1:25 with assay buffer to obtain the highest concentration used in the standard curve (20ng/ml). *Suggestion:* Add 40µl of the 500ng/ml stock solution to 960µl Assay Buffer to obtain a 20ng/ml solution. Dilute this 20ng/ml solution further down in six separate 1.5 - 2ml polypropylene tubes. *Suggestion:* Make a serial 1/2-dilution by repeated transfer of 1 part (e.g. 400µl) standard solution into 1 part (e.g. 400µl) Assay Buffer.

Store unused portion of stock solution at -20°C for further use.

Often, the two highest standards can be omitted since MRP14 levels in serum are generally low.

Detection reagent:

Dissolve reagent S-1008C in 500µl water to give a **30µg/ml** stock solution. Incubate a few minutes to allow complete dissolution. 200µl of this stock solution are diluted in 20ml Assay Buffer to give a concentration of 0.3µg/ml. Add 20µl of the enclosed Streptavidin-Peroxidase conjugate, mix gently and incubate for 10 minutes at room temperature to obtain the working solution.

Store unused portion of the stock solution at -20°C until further use.

Substrate dilution:

Prepare required quantity immediately before use. For one whole plate (12 strips): Mix 20ml substrate buffer (S-1008F) with 1ml substrate stock solution (S-1008E). This dilution should be used 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. Do not add the water to the acid**). 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 Blocking Reagent to each well.
- 1a. Add 100µl each of the 20ng/ml standard to wells A1 and A2, and the respective dilutions down the column as suggested below. Wells H1 and H2 are blanks and get 100µl Assay Buffer.
- 1b. Add 100µl of appropriately diluted sample to the corresponding wells.
2. Incubate for 45 minutes at 37°C.
3. Wash the plate 4 times with PBS or isotonic saline, preferably in an automatic plate washer. Empty the plate and blot it onto a soft absorbent paper.
4. Add 200µl diluted detection reagent (working dilution including extravidin-peroxidase) to each well and incubate at 37°C for 1 hour.
5. Wash plate as in step 3 and blot plate onto a soft absorbing paper to eliminate remaining water. Make sure that STOP solution for step 7 is ready.
6. Add 200µl diluted substrate (citrate buffer with TMB) per well with a multichannel dispenser, incubate for **2 minutes** at room temperature.
7. Stop color reaction with 100µl STOP solution per well. Coloration turns from blue to yellow. **Caution:** Stop solution contains 1N sulfuric acid which is corrosive and causes burns.
8. Read absorbance within one hour at 450nm with reference wavelength set to 630nm, if possible.

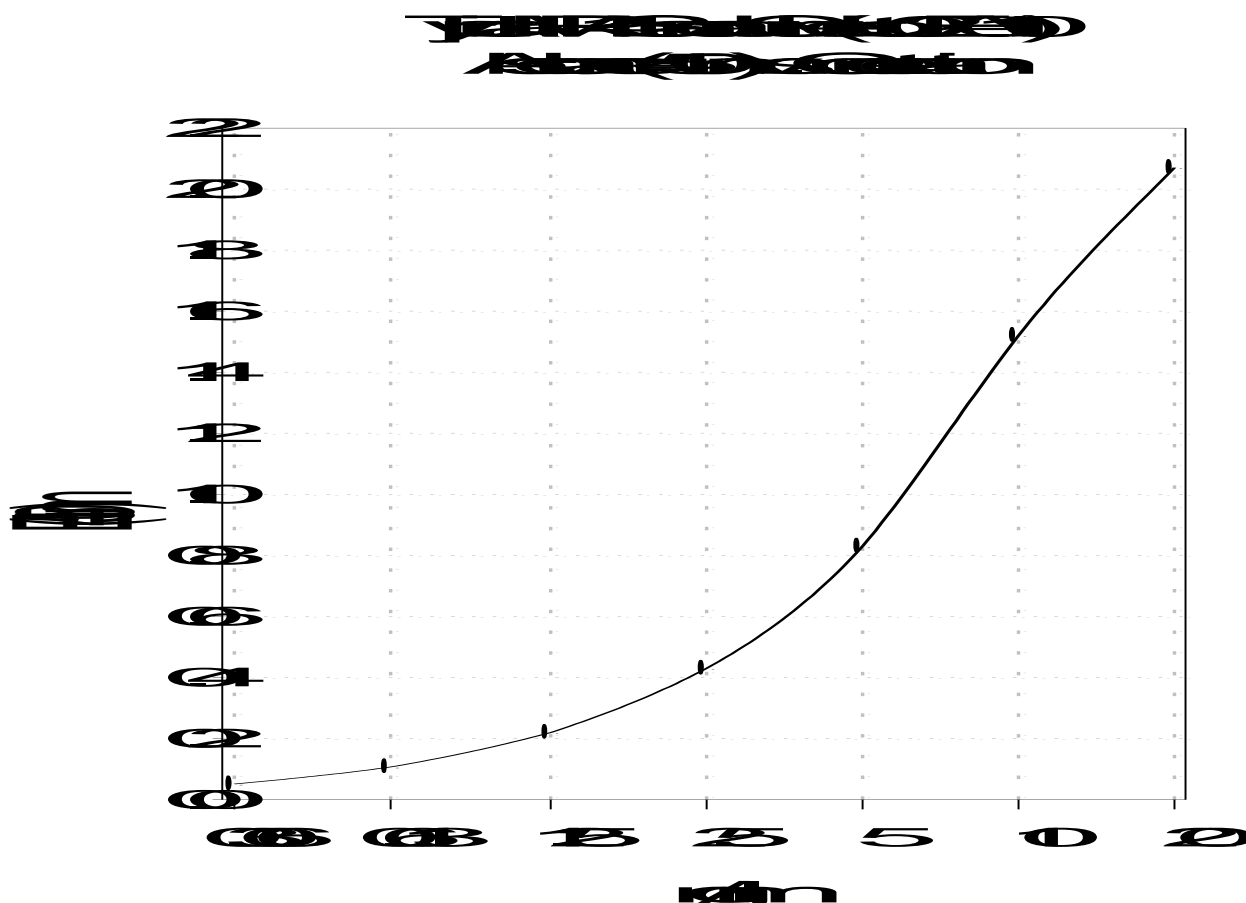
Suggested plate set-up:

The following plate arrangement may be chosen, where St20 through St0.31 are the standard dilutions from 20ng/ml to 0.31ng/ml, as described above. Sa-1 through Sa-38 are samples in duplicates. Cont refers to a control serum with a known MRP14 content (not included in the kit).

	1	2	3	4	5	6	7	8	9	10	11	12
A	St20	St20	Sa-1	Sa-1	Cont	Cont	Sa-16	Sa-16	Sa-24	Sa-24	Sa-32	Sa-32
B	St10	St10	Sa-2	Sa-2	Sa-9	Sa-9	Sa-17	Sa-17	Sa-25	Sa-25	Sa-33	Sa-33
C	St5	St5	Sa-3	Sa-3	Sa-10	Sa-10	Sa-18	Sa-18	Sa-26	Sa-26	Sa-34	Sa-34
D	St2.5	St2.5	Sa-4	Sa-4	Sa-11	Sa-11	Sa-19	Sa-19	Sa-27	Sa-27	Sa-35	Sa-35
E	St1.25	St1.25	Sa-5	Sa-5	Sa-12	Sa-12	Sa-20	Sa-20	Sa-28	Sa-28	Sa-36	Sa-36
F	St0.63	St0.63	Sa-6	Sa-6	Sa-13	Sa-13	Sa-21	Sa-21	Sa-29	Sa-29	Sa-37	Sa-37
G	St0.31	St0.31	Sa-7	Sa-7	Sa-14	Sa-14	Sa-22	Sa-22	Sa-30	Sa-30	Sa-38	Sa-38
H	Blank	Blank	Sa-8	Sa-8	Sa-15	Sa-15	Sa-23	Sa-23	Sa-31	Sa-31	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

Complex formation of the MRP14 complex may be influenced by the presence of Ca^{2+} . The assay buffer contains CaCl_2 . We advise not to use phosphate buffers because of precipitation of insoluble calcium phosphate (solubility products: CaHPO_4 $\text{pK}_{\text{sp}} = 7.0$ and $\text{Ca}_3(\text{PO}_4)_2$ $\text{pK}_{\text{sp}} = 28.7$).

Normal values for EDTA plasma (10mM EDTA) or serum are practically identical. Heparin plasma may yield higher values.

Room temperature and incubation time affect all the reactions more or less. If it happens that the highest (20ng/ml) standard value is off scale ($E^{450\text{nm}} > 3.0$), we suggest to remove it altogether from your standard curve since most samples yield low values. Alternatively, remove the same volume of solution from every well (e.g. 100 μl) and immediately 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 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 – 3000 ng/mL
MRP8	< 50 ng/mL
MRP14	< 5 ng/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:

- High MRP8 concentration
- 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.

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