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MRP8/14 (Calprotectin) Enzyme Immunoassay

Test for the determination of MRP8/14 in biological fluids

Test Instructions

Product Code: S-1011

Kit contains: 2 precoated, dry 12-strip microtiter plates and reagents to perform 2x96 tests. Refrigerate upon arrival.

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Contents

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 MRP8/14 (or MRP8) levels with disease activity:

- MRP8/14 levels in stool are a reliable indicator of intestinal inflammatory conditions.
- Plasma MRP8/14 level can be a marker 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 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.

Typical MRP patterns have been reported 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

One-step non-competitive sandwich assay using a highly specific monoclonal antibody for antigen capture, and a polyclonal chicken antibody for detection. Reagent limited with peroxidase catalyzed tetramethylbenzidin color reaction, including a stop reaction and reading at 450/630nm in a multititer plate reader.

Reagents provided

S-1011A:	Ready-to-use precoated and stabilized microtiter plates (Nunc Maxisorp) + plate sealer (2 each per kit).
S-1011B	1.25ml of a 1000ng/ml standard, a preparation of MRP8/14 complex. Dilute with assay buffer to obtain the desired concentrations for a standard curve.
S-1011C	65µl of a peroxidase conjugated detection antibody. Spin briefly before opening to retrieve all the solution
S-1011D	60ml assay buffer (3x concentrated), a slightly turbid solution containing a blocking agent and a germicide.
S-1011E	2.2ml Tetramethylbenzidine (TMB) – H_2O_2 solution. Keep dark.
S-1011F	60ml substrate buffer (potassium citrate)

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

Try to estimate how much of the various solutions you will need and prepare the actual volumes that will be required in one experiment. Duplicate testing of samples and standards is highly recommended. Allow the reagents to warm up to room temperature and prepare the following dilutions immediately before use:

Assay Buffer:
Dilute the concentrated green stock solution S-1011D with two parts purified water. Example: add 30ml water to 15ml stock solution.

Samples:
Store samples in aliquots at –20°C or lower. Use an aliquot only once and dilute it in appropriately diluted assay buffer (S-1011D). Recommended dilutions are 1:100 for serum or plasma; 1:100 - 1:10,000 for synovial fluid; 1:10 for exhalates, saliva, BAL and urine.

Standards:
The 1000ng/ml stock solution of MRP8/14 is vortexed gently and ready to be diluted appropriately as indicated below. Store this solution at 4° - 8°C for several weeks or in aliquots at -20°C for further use, if necessary.

Detection reagent (S-1011C):
Dilute reagent S-1011C with assay buffer to give a 1:400 dilution. Example: add 25µl to 10ml assay buffer. 10ml are sufficient for one 96-well plate

Substrate dilution:
To be prepared immediately before use. Bring substrate buffer (S-1011F) to room temperature before use. The dilution is prepared by mixing 20 parts substrate buffer with 1 part TMB - H_2O_2 solution (S-1011E). This dilution should be used within 15 minutes after preparation.

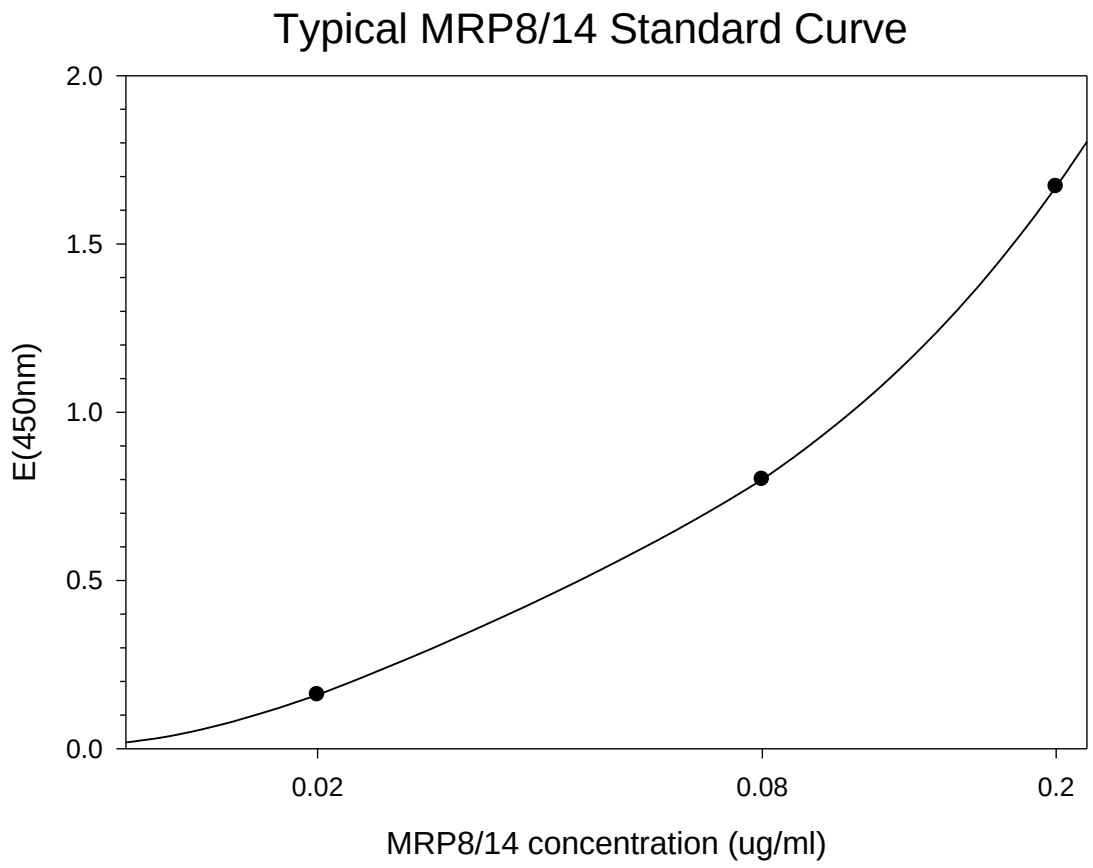
STOP solution:
Dilute sulfuric acid to a concentration of **1N**. Example: Add 2.9ml 95-97% sulfuric acid to 100ml water (In this sequence. Do not add the water to the acid). 95-97% concentrated sulfuric acid (specific gravity 1.84) is 36N.

Test procedure

1. The standard should be diluted in separate glass or polypropylene tubes (do not use polystyrene tubes). Prepare the following dilutions:
   a) Pipet 400µl assay buffer (each) in three separate tubes. Mark them as 200ng/ml, 80ng/ml and 20ng/ml.
   b) Add 100µl of the 1000ng/ml standard to the first tube, mix gently (= 200ng/ml).
   c) Add 34.5µl of the 1000ng/ml standard to the second tube, mix gently (= 80ng/ml).
   d) Add 8.1µl of the 1000ng/ml standard to the third tube, mix gently (= 20ng/ml)
   e) Add 100µl of each of the three standard solutions to the corresponding wells in the microtiter plate (see plate set-up below).
   f) Add 100µl assay buffer in the wells reserved for the blanks (see below).
2. Add 100µl of appropriately diluted sample to the corresponding wells.
3. Add 100µl diluted detection reagent S-1011C (1:400, see above) to each well and incubate over night (15 - 17 hours) at 4-8°C (suggested protocol) or 2 hours at 37°C with shaking (300rpm, fast protocol) or 6 hours at room temperature (intermediate protocol).
4. After incubation, wash wells with water (6 times) or isotonic buffer and blot plate onto a soft absorbing paper to eliminate remaining water. Tap water of drinking quality (non chlorinated) or purified water can be used, as well as PBS or istonic saline (0.9% NaCl).
5. Immediately add 200µl substrate dilution (see above) to each well. Incubate for 6 -8 (six to eight) minutes at room temperature. A blue color reaction occurs where MRP8/14 is present.
6. Stop color reaction by adding 100µl stop solution to each well. Coloration turns from blue to yellow.
7. Read absorbance within one hour at 450nm with reference wavelength set to 630-650nm.

Suggested plate set-up:
As an example, the following plate arrangement can be chosen, where St200 through St20 are the standard dilutions from 200ng/ml to 20ng/ml, as described above. Sa-1 through Sa-42 are samples in duplicates. Cont refers to a control serum with a known MRP8/14 content (not included in the kit).

	1	2	3	4	5	6	7	8	9	10	11	12
A	St200	St200	Sa-5	Sa-5	Cont	Cont	Sa-20	Sa-20	Sa-28	Sa-28	Sa-36	Sa-36
B	St80	St80	Sa-6	Sa-6	Sa-13	Sa-13	Sa-21	Sa-21	Sa-29	Sa-29	Sa-37	Sa-37
C	St20	St20	Sa-7	Sa-7	Sa-14	Sa-14	Sa-22	Sa-22	Sa-30	Sa-30	Sa-38	Sa-38
D	Blank	Blank	Sa-8	Sa-8	Sa-15	Sa-15	Sa-23	Sa-23	Sa-31	Sa-31	Sa-39	Sa-39
E	Sa-1	Sa-1	Sa-9	Sa-9	Sa-16	Sa-16	Sa-24	Sa-24	Sa-32	Sa-32	Sa-40	Sa-40
F	Sa-2	Sa-2	Sa-10	Sa-10	Sa-17	Sa-17	Sa-25	Sa-25	Sa-33	Sa-33	Sa-41	Sa-41
G	Sa-3	Sa-3	Sa-11	Sa-11	Sa-18	Sa-18	Sa-26	Sa-26	Sa-34	Sa-34	Sa-42	Sa-42
H	Sa-4	Sa-4	Sa-12	Sa-12	Sa-19	Sa-19	Sa-27	Sa-27	Sa-35	Sa-35	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 MRP8/14 heterocomplex needs the presence of Ca^{2+}. Assay buffer contains 5 mmol/L $CaCl_2$. We advise not to use phosphate buffers because of precipitation of insoluble calcium phosphate (solubility products: $CaHPO_4$ pK_{sp} = 7.0 and $Ca_3(PO_4)_2$ pK_{sp} = 28.7).

The Ca^{2+} in the MRP8/14 heterocomplex is bound very tightly and remains compelxed in plasma in the presence of EDTA. Thus the normal values for EDTA plasma or serum are practically identical. Heparin plasma yields higher values, possibly due to the release of MRP8/14 from neutrophils.

The tap water used for washing the plates may contain high chlorine levels which negatively affect the performance.

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 – 3500 ng/ml
MRP8	< 50 ng/ml
MRP14	< 2 ng/ml

The concentration of the MRP8/14 complex is an indication of the severity of inflammation (extreme values of >>10,000ng/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 may indicate 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 and
- A 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
- Reasonable concentration of the MRP8/14 heterocomplex (approx. 3,000 ng/mL). The MRP8/14 concentration is elevated (4,000-100,000 ng/mL serum) in an acute phase of chronic inflammation.

Viral infections alone do not result in elevated MRP8/14 concentrations. 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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