BÜHLMANN MRP8/14

ELISA

Calprotectin S100A8/A9

For Research Use Only.
Not for use in diagnostic procedures.

EK-MRP8/14-U 96 tests

Revision date: 2015-10-28

BÜHLMANN LABORATORIES AG
Baselstrasse 55
CH - 4124 Schönenbuch, Switzerland
Tel.: +41 61 487 1212
Fax: +41 61 487 1234
info@buhlmannlabs.ch

ENGLISH

INTENDED USE

The BÜHLMANN MRP8/14 ELISA kit is designed for the quantitative determination of the heteropolymer of myeloid-related protein MRP8/14 (Calprotectin, S100A8/S100A9) in serum (1-4).
For Research Use Only. Not for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

The test allows the selective measurement of MRP 8/14-antigen by sandwich ELISA. A monoclonal capture antibody (mAb) highly specific to the MRP8/14 heterodimeric and polymeric complexes (5-7), respectively, is coated onto the microtiter plate. A second monoclonal detection antibody (Ab) conjugated to horseradish peroxidase (HRP) detects the MRP8/14 molecules bound to the monoclonal antibody coated onto the plate after a washing step. After incubation and a further washing step, tetramethylbenzidine (TMB) will be added (blue color formation) followed by a stopping reaction (change to yellow color). The absorption is measured at 450 nm.

REAGENTS SUPPLIED AND PREPARATION

Reagents	Quantity	Code	Reconstitution
Microtiter Plate precoated with anti-MRP8/14 mAb	12 x 8-wells	B-MRP8/14-MP	Ready to use
Plate Sealer	3 pieces		
Wash Buffer Concentrate (10x) with preservatives	1 bottle 100 ml	B-MRP8/14-WB	Dilute with 900 ml of deionized water
Incubation Buffer with preservatives	1 bottle 100 ml	B-MRP8/14-IB	Ready to use
Calibrators A to E*) MRP8/14 in a buffer matrix with preservatives	5 vials 1 ml	B-MRP8/14-CASET	Ready to use
Control Low / High Human serum matrix with preservatives	2 vials 1 ml	B-MRP8/14-CONSET	Ready to use.
Enzyme Label Anti-MRP8/14 Ab conjugated to HRP	1 vial 11 ml	B-MRP8/14-EL	Ready to use
TMB-Substrate TMB in citrate buffer with H_2O_2	1 vial 11 ml	B-TMB	Ready to use
Stop Solution 0.25 M sulfuric acid	1 vial 11 ml	B-STS	Ready to use **Corrosive agent**

Table 1

*) The actual concentration of the standards A to E are 4, 12, 40, 120 and 240 ng/ml MRP8/14, respectively. Serum samples will be diluted 1:100, therefore the calibrators A-E are labeled as follows: 0.4, 1.2, 4.0, 12.0 and 24.0 µg/ml.

STORAGE AND SHELF LIFE OF REAGENTS

Unopened Reagents	
Store at 2-8°C. Do not use past kit expiration date printed on the labels.	
Opened / Reconstituted Reagents	
Microtiter Plate	Return unused strips immediately to the foil pouch containing the desiccant packs and reseal along the entire edge of zip-seal. Store until expiration date at 2-8°C.
Diluted Wash Buffer	Store for up to 6 months at 2-8°C.
Incubation Buffer	Store at 2-8°C until expiration date.
Controls	
Calibrators	
Enzyme Label	
TMB-Substrate	
Stop Solution	

Table 2

PRECAUTIONS

SAFETY PRECAUTIONS

- The microtiter-plate, calibrators and controls of this test contain components of human origin. Although tested and found negative for HBV surface antigen, HCV and HIV1/2 antibodies, the reagents should be handled as if capable of transmitting infections and should be handled in accordance with good laboratory practices using appropriate precautions.
- **Substrate and Stop Solution**: Substrate and Stop Solution: The Substrate TMB (B-TMB) contains Tetramethylbenzidine, hydrogen peroxide (H2O2) and dimethylformamide. The Stop Solution (B-STS) contains sulfuric acid (0.25 M). Each of those reagents is irritant to eyes, skin and mucous membranes. Avoid contact with Eyes, skin and cloths Wear suitable protective clothing, gloves and eye protection. After contact with eyes or skin, wash immediately with plenty of water.
- Unused solution should be disposed of according to local State and Federal regulations.

TECHNICAL PRECAUTIONS

Kit components

- **Residues in the microtiter plate** wells result from the production process. They are removed in the washing step (Assay procedure step 3) and do not affect the results.
- Read carefully the instructions prior to carrying out the test. Test performance will be adversely affected, if reagents are incorrectly diluted, modified or stored under conditions other than those as detailed in this instruction for use.
- Components must not be used after the expiry date printed on the labels.
- Do not mix different lots of reagents.
- Every effort should be made to ensure that no cross contamination occurs between reagents, samples or between wells.
- Let the reagents adjust to reach room temperature. Mix well (vortex) the reagents before use.
- Microwells cannot be re-used.

ELISA Procedure:

- In the ELISA procedure the **washing step is essential** to guarantee reproducible results. A **minimal incubation time** of the Wash Buffer in the wells **of at least 20 seconds** must be ensured each time. The indicated no. of **washing cycles is mandatory** to ensure reproducible results.
- When using **automated washer,** BÜHLMANN strongly recommends using “plate mode” i.e. each process step (dispense/aspiration) is performed on all of the strips sequentially, before processing to the next process step. Thus, the minimal incubation time is guaranteed.
- The indicated **washing cycles are mandatory** to ensure reproducible results.
- To ensure a complete antigen/antibody interaction, the **incubation time in step 5** should not be less than 30 minutes. Moderately longer incubation time has no influence to the final outcome.
- The enzyme used as the label is inactivated by oxygen and is highly sensitive to sodium azide, thimerosal, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Therefore, use only deionized high quality water.

- It is recommended to assay each control and specimen in **duplicate** each time a test is performed. Since conditions vary from assay to assay, a new standard curve must be generated each time a new assay is performed. Vertical alignment is recommended.
- If the initial concentration of an unknown sample reads greater than the highest Calibrator (Calibrator E), the sample must be further diluted with Incubation Buffer and assayed again according to the assay procedure. The resulting dilution factor must be accounted for the final calculation.

MATERIALS REQUIRED BUT NOT PROVIDED

- 10 µl, 100 µl and 1000 µl precision pipettes with disposable tips.
- Disposable polystyrene or polypropylene tubes for the preparation of sample dilutions.
- 1000 ml cylinder for the dilution of the Wash Buffer Concentrate.
- Microtiter plate washer or squeeze bottle for Wash Buffer.
- Microtiter plate rotator.
- Blotting paper.
- Microtiter plate reader for measurement of absorbance at 450 nm.

STANDARDIZATION

The test is calibrated against purified MRP8/14 from human granulocytes.

SPECIMEN COLLECTION AND STORAGE

Less than 10 µl serum are needed for the quantification of samples in duplicates.

Serum: Collect blood into plain tubes, avoid hemolysis, mix by gentle inverting sample tubes several times and leave to clot for 45 minutes at room temperature (18-28°C) protected from light. Centrifuge at 1800 x g for 15 minutes at room temperature (18-28°C) and collect the serum immediatelly. Do not heat-inactivate samples. Samples may be stored at 2-8°C for up to 30 days. If samples have to be stored for a longer period, they are stable at ≤-20°C for at least 6 months.

Lipemic, hemolytic and icteric samples should not be used in this assay. Lipemic samples can be avoided by asking patients to fast for at least 12 hours prior to drawing the sample.

Note: MRP8/14 measurement in plasma from heparinized or citrated blood samples is possible. Be aware that normal values are slightly effected by the sample type used.

ASSAY PROCEDURE

Allow the reagents to equilibrate to 18-28°C prior to use

1. Dilute all patient serum samples 1:100 with Incubation Buffer (e.g. 10 µl serum and 990 µl incubation buffer) and mix well. Allow diluted samples to stand for 15 minutes at 18-28°C prior to pipetting in step 4c.
2. Prepare a plate with sufficient strips to test the required number of calibrators, controls and samples. Remove excess strips from the holder and re-seal them in the foil pouch together with the desiccant packs **without delay**. Store refrigerated.
3. Wash the coated wells twice using at least 300 µl of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.

4a. Pipet 100 µl of Incubation Buffer in duplicate into wells A1+A2 (Blank).
Pipet 100 µl of Calibrator A in duplicate into wells B1+B2.
Pipet 100 µl of Calibrator B in duplicate into wells C1+C2 etc.

4b. Pipet 100 µl of the Low and High Serum Control in duplicate into wells G1+G2 and H1 and H2, respectively.

4c. Pipet 100 µl of each diluted sample in duplicate into the subsequent wells.

5. Cover the plate with a plate sealer, place the plate on a plate rotator set at 400–600 rpm and incubate for 30 minutes at 18-28°C (see Procedural Notes).
6. Remove and discard the plate sealer. Empty the wells and wash three times using at least 300 µl of Wash Buffer per well (see Procedural Notes). Empty the wells and strike the plate firmly onto blotting paper.
7. Pipet 100 µl of Enzyme Label to all wells.
8. Cover the plate with a plate sealer, place the plate on a plate rotator set at 400-600 rpm and incubate for 30 ± 5 minutes at 18-28°C.
9. Remove and discard the Plate Sealer. Empty the wells and **wash five times** using at least 300 µl of Wash Buffer per well. Empty the wells and strike the plate firmly onto blotting paper.

Important: Allow the TMB substrate solution to reach 18-28°C.

10. Pipet 100 µl of the TMB Substrate Solution to all wells.
11. Cover the plate with a plate sealer, place the plate on a plate rotator set at 400-600 rpm, protect the plate from direct light and incubate for 15 ± 2 minutes at 18-28°C.
12. Pipet 100 µl of Stop Solution to all wells. Remove air bubbles with a pipette tip. Proceed to step 13. within 30 minutes.
13. Read the absorbance at 450 nm by using a microtiter plate reader.

RESULTS

Standard Curve: Record the absorbance at 450 nm for each calibrator and blank well. Average the duplicate values, subtract the average of the blank wells and record averages (=corrected average absorbance). Plot the absorbance (vertical axis) versus the MRP8/14 concentration of the calibrators (horizontal axis) using a semi logarithmic lin/log graph. Draw the best fitting curve or calculate the standard curve using a four parameter logistic.
Samples and Controls: Record the absorbance at 450 nm for each sample and control well. Average the duplicate values, subtract the average of the blank wells and record the averages (=corrected average absorbance). Locate the corrected absorbance value of the sample on the vertical axis, draw a horizontal line intersecting the standard curve and read the MRP8/14 concentration from the horizontal axis. **The results already include the dilutions done during the procedure.**

See Table 3 and Figure 1 for typical data (results and standard curve). *These results and standard curve are provided for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.*

QUALITY CONTROL

A thorough understanding of this instruction for use is necessary for the successful use of the product. Reliable results will be obtained only by using precise laboratory techniques (current GLP guidelines) and accurately following this instruction for use.

Since there is no control serum for MRP8/14 commercially available, we recommend using a positive pool for internal quality control.

The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. The confidence limits for the Controls are lot-specific and printed on the additional QC data sheet.

If the precision of the assay does not correlate with the established limits and repetition excludes errors in technique, check the following issues: i) pipetting, temperature controlling and timing devices ii) ELISA reader settings iii) expiration dates of reagents iv) storage and incubation conditions v) TMB Substrate Solution should be colorless vi) purity of water.

PERFORMANCE LIMITATIONS

Test results should be interpreted in conjunction with information available from clinical assessment of the patient and other diagnostic procedures.

PERFORMANCE CHARACTERISTICS

Intra-Assay Precision: 4.3 %. The intra-assay precision was calculated from the results of 20 pairs of values from 7 different serum samples each obtained in a single run. Each serum sample was assayed according to the assay procedure. The values are presented in Table 4.

Inter-Assay Precision: 5.8 %. The inter-assay precision was calculated from two EDTA plasma and serum samples, each in 20 different runs. Each plasma and serum sample was assayed according to the assay procedure. The values are presented in Table 5.

Detection limit (LoB): <<0.4 µg/ml. Twenty duplicates of Incubation Buffer were assayed in a single run. Mean and standard deviation were calculated for the absorbance values. The minimal detectable dose of MRP8/14 was calculated to be far below 0.4 µg/ml (Calibrator A) by adding two standard deviations to the mean absorbance and intersecting this value with the standard curve obtained in a new run.

Detection limit (LoQ): < 0.4 µg/ml. Eight different serum samples with values between 0.21 and 39.8 µg/ml MPR8/14 were assayed 20 times in duplicates as an intra-assay. The %CV and the mean values were calculated for each sample. The functional sensitivity was observed at 10% CV. The resulting precision profile (Figure 2) allows the precise measurement within the entire Standard range from 0.4 to 24 µg/ml.

Dilution Linearity: 97.8%. Two human EDTA plasma and serum samples each, with elevated MRP8/14 values were diluted with Incubation Buffer and subsequently assayed according to the protocol. The expected values were calculated from the observed value found with the first dilution. The values are presented in Table 6.

Spiking Recovery: 100.8%. Two EDTA plasma and serum samples each, were assayed before and after spiking with different amounts of human MRP8/14. The values are presented in Table 7.

Crossreactivity: <0.1%. Incubation Buffer spiked with different amounts of recombinant monomer MRP8 and MRP14 were measured according to the assay procedure, respectively. The values are presented in Table 8.

Table 3: **Example of Results**

	MRP8/14 Conc. [µg/ml]	Absorb. [OD]	CV Conc [%]
Blank Avg.		**0.060**	
Cal A	0.4	0.073	
Cal A	0.4	0.070	
Cal A Avg.	**0.4**	**0.072**	**2.2**
Cal B	1.2	0.217	
Cal B	1.2	0.216	
Cal B Avg.	**1.2**	**0.217**	**0.3**
Cal C	4.0	0.669	
Cal C	4.0	0.686	
Cal C Avg.	**4.0**	**0.678**	**1.7**
Cal D	12.0	1.563	
Cal D	12.0	1.547	
Cal D Avg.	**12.0**	**1.555**	**0.7**
Cal D	24.0	2.199	
Cal D	24.0	2.167	
Cal D Avg.	**24.0**	**2.183**	**1.0**
Ctrl Low		0.303	
Ctrl Low		0.302	
Ctrl Low Avg.	**1.7**	**0.303**	**0.1**
Ctrl High		0.997	
Ctrl High		0.958	
Con High Avg.	**6.2**	**0.978**	**3.5**
Serum 1		1.920	
Serum 1		1.881	
Serum 1 Avg.	**17.4**	**1.901**	**3.0**
Plasma 1		0.157	
Plasma 1		0.165	
Plasma 1 Avg.	**0.9**	**0.161**	**3.5**

Figure 1: **Example of a Standard Curve**

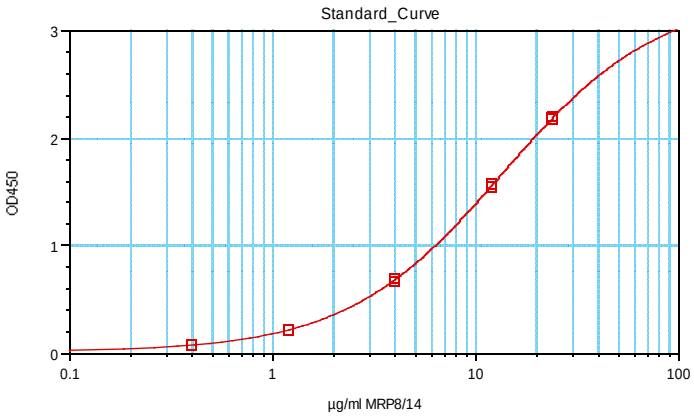

Table 4: **Intra-Assay Precision (Within-Run)**

Sample	Mean [µg/ml]	SD [µg/ml]	CV [%]
Serum 1	21.64	0.77	3.6
Serum 2	7.46	0.29	3.9
Serum 3	5.76	0.18	3.1
Serum 4	1.46	0.04	2.4
Serum 5	1.01	0.03	3.0
Serum 7	0.51	0.03	6.4
	0.21	0.02	7.4
Mean			**4.3**

Table 5: **Inter-Assay Precision (Run-to-Run)**

Sample type	Mean [µg/ml]	SD [µg/ml]	CV [%]
Serum 8	12.1	0.7	5.6
Serum 9	8.2	0.4	4.7
Plasma 1	3.7	0.2	5.5
Plasma 2	0.9	0.1	7.4
Mean			**5.8**

Figure 2: **Precision Profile**

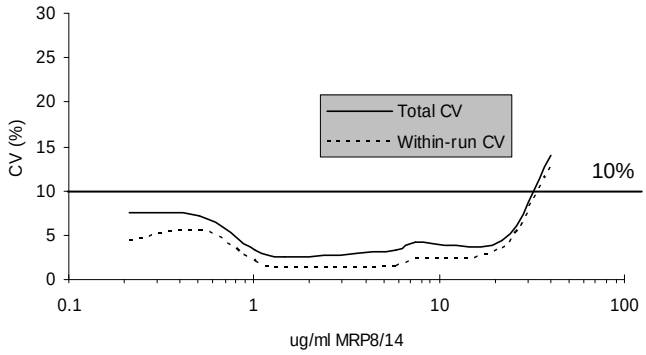

Table 6: **Dilution Linearity/Parallelism**

Sample	Dilution	Observed [µg/ml]	Expected [µg/ml]	O/E [%]
Serum 10	1:100	23.08	-	--
	1:200	10.76	11.54	93.2
	1:400	4.95	5.77	85.8
	1:800	2.52	2.89	87.3
	1:1600	1.32	1.44	91.5
	1:3200	0.61	0.72	84.6
Serum 11	1:100	7.92	-	--
	1:200	4.10	3.96	103.5
	1:400	2.18	1.98	109.8
	1:800	1.08	0.99	108.6
	1:1600	0.53	0.50	106.7
Plasma 3	1:100	2.49	-	--
	1:200	1.22	1.25	98.0
	1:400	0.60	0.62	96.4
Plasma 4	1:100	4.41	-	--
	1:200	2.20	2.21	99.8
	1:400	1.11	1.10	100.7
	1:800	0.53	0.55	96.1
Mean				**97.8**

Table 7: **Spiking Recovery**

Sample	Basic Value [µg/ml]	Spiked with [µg/ml]	Observed [µg/ml]	Expected [µg/ml]	O/E [%]
Serum 12	0.81	0.2	0.96	1.01	95.0
		1.0	1.95	1.81	107.7
		2.0	2.86	2.81	101.8
		4.0	4.99	4.81	103.7
Serum 13	1.00	0.2	1.03	1.2	85.8
		1.0	1.95	2.0	97.5
		2.0	2.90	3.0	96.7
		4.0	5.44	5.0	108.8
Plasma 5	1.20	0.2	1.42	1.4	101.4
		1.0	2.51	2.2	114.1
		2.0	3.30	3.2	103.1
		4.0	5.30	5.2	101.9
Plasma 6	2.12	0.2	2.29	3.32	98.7
		1.0	3.17	3.12	101.6
		2.0	3.96	4.12	96.1
		4.0	6.01	6.12	98.2
Mean					**98.7**

Figure 3: **Comparison Serum vs. EDTA Plasma**

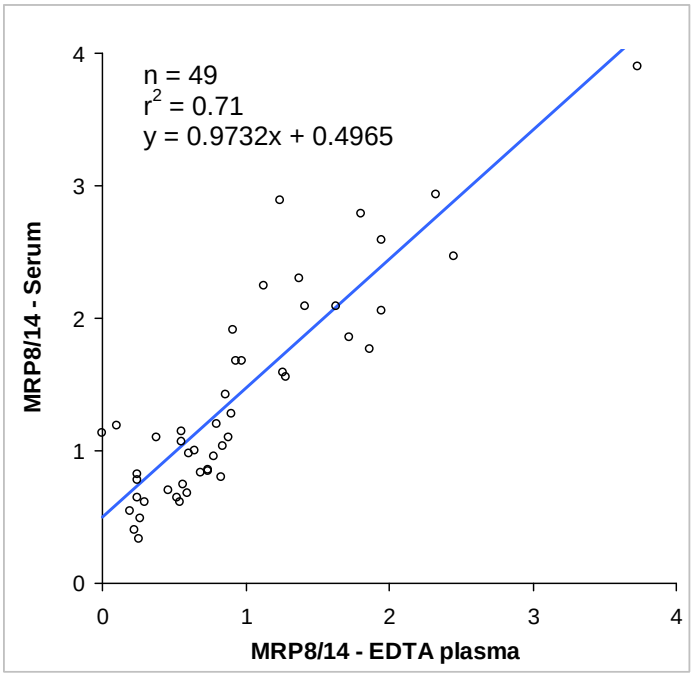

Table 8: **Cross Reactivity**

Spiked with MRP8	% detected in EK-MRP8/14-U
100 µg/ml	0.05
10 µg/ml	0.01
1 µg/ml	<0.01
100 ng/ml	<0.01
10 ng/ml	<0.01
1 ng/ml	<0.01

Spiked with MRP14	% detected in EK- MRP8/14
100 µg/ml	0.09
10 µg/ml	0.02
1 µg/ml	<0.01
100 ng/ml	<0.01
10 ng/ml	<0.01
1 ng/ml	<0.01

APPENDIX II
REFERENCES

1. Altwegg L.A. *et al*. *Myeloid-related protein 8/14 complex is released by monocytes and granulocytes at the site of coronary occlusion: a novel, early, and sensitive marker of acute coronary syndromes*. Eur Heart J **28(8)**,941-8 (2007)
2. Healy A.M. *et al. Platelet expression profiling and clinical validation of myeloid-related protein 14 as a novel determinant of cardiovascular events.* Circulation **113**, 2278-84 (2006)
3. Burkhardt, K.*, et al. An increase in myeloid-related protein serum levels precedes acute renal allograft rejection.* J Am Soc Nephrol **12**, 1947-57 (2001).
4. Striz, I.*, et al. MRP 8/14 and procalcitonin serum levels in organ transplantations.* Ann Transplant **6**, 6-9 (2001)
5. Hessian, P.A. and Fisher, L. *The heterodimeric complex of MRP-8 (S100A8) and MRP-14 (S100A9). Antibody recognition, epitope definition and the implications for structure*. Eur J Biochem **268**, 353-63 (2001).
6. Goebeler, M.*, et al. Expression and complex formation of S100-like proteins MRP8 and MRP14 by macrophages during renal allograft rejection.* Transplantation **58**, 355-61 (1994).
7. Zwadlo et al. *A monoclonal antibody to a subset of human monocytes found only in the peripheral blood and inflammatory tissues.* J Immunol **137**, 512-8 (1986)

APPENDIX III
SHORT PROTOCOL

MRP8/14 ELISA

Precoated Microtiter Plate

wash 2 x

100 µl Calibrators, Controls or diluted Samples

incubate 30 ± 5 min at 18-28°C on a plate rotator

wash 3 x (see procedural notes)

add 100 µl Enzyme Label

incubate 30 ± 5 min. at 18-28°C on a plate rotator

wash 5 x *(see procedural notes)*

add 100 µl TMB Substrate

incubate 15 ± 2 minutes at 18-28°C on a plate rotator

add 100 µl Stop Solution

➡ **Read absorbance at 450 nm (within 30 minutes)**

TIME TO RESULT: 75 MINUTES

APPENDIX IV
SYMBOLS/ SYMBOLE/ SYMBOLES

Symbol	Explanation
	Use By
REF	Catalogue number
LOT	Batch code
	Contains sufficient for <n> tests
	Consult Instructions for Use-
	Temperature limitation
MP	Microtiterplate

Symbol	Explanation
[BUF]WASCH]10X]	Wash Bufer Concentrate (10x)
[BUF]INC]	Incubation Buffer
[CAL[A] – [CAL[E]	Calibrator A-E
[CONTROL[S[L]	Control Serum Low
[CONTROL[S[H]	Control Serum High
[EL]	Enzyme Label
[SUBS[TMB]	TMB Substrate
[SOLN]STOP]	Stop Solution

Printing Date
2015-12-07