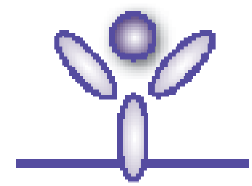
LEGEND MAX™

ELISA Kit



Human MRP8/14 (Calprotectin)

Cat. No. 439707

ELISA Kit for Accurate Quantitation of Human MRP8/14 (Calprotectin) from Cell Culture Supernatant, Serum, Plasma, Urine, Saliva and Other Biological Fluids

BioLegend, Inc.
biolegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

For Research Purposes Only. Not for use in diagnostic or therapeutic procedures. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of BioLegend is strictly prohibited.

Table of Contents

Page

Introduction.......... 2
Materials Provided.......... 3
Materials to be Provided by the End-User.......... 3
Storage Information.......... 4
Health Hazard Warnings.......... 4
Specimen Collection and Handling.......... 5
Reagent and Sample Preparation.......... 5
Assay Procedure.......... 6
Assay Procedure Summary.......... 8
Calculation of Results.......... 9
Typical Data.......... 9
Performance Characteristics.......... 9
- Specificity.......... 9
- Sensitivity.......... 9
- Recovery.......... 10
- Linearity.......... 10
- Intra-Assay Precision.......... 10
- Inter-Assay Precision.......... 10
- Biological Samples.......... 10

Troubleshooting Guide.......... 11
ELISA Plate Template.......... 13

Introduction:

Migration inhibitory factor-related proteins MRP8 (S100A8) and MRP14 (S100A9) are members of the S100 family of calcium binding proteins. They are highly expressed in resting neutrophils, keratinocytes, in infiltrating tissue macrophages, and on epithelial cells in active inflammatory disease. Human MRP8 has a molecular weight of 11.0 kD, while human MRP14 exists in a 13.3 kD and a truncated 12.9 kD form. Calcium induces the formation of MRP8/14 hetero-complexes (Calprotectin). MRP8/14 complexes are produced in large quantity by activated granulocytes and monocytes.

MRP8, MRP14, and their heterodimer MRP8/14 have been shown to have major roles in inflammatory and immunological responses. MRP8 and MRP14 regulate myeloid cell function by binding to Toll-like receptor-4 and the receptor for advanced glycation end-products (AGE). One major function of the MRP8/14 heterocomplex is its antimicrobial activity (hence the name calprotectin) by inhibiting the growth of pathogens through competition for zinc. MRP8/14 also regulates vascular inflammation and contributes to the biological response to vascular injury by promoting leukocyte recruitment, macrophage cytokine production, and SMC proliferation. In addition, MRP8/14 hetero-complex has a unique role as a fatty acid transport protein. This makes MRP8/14 an important mediator between calcium signaling and arachidonic acid effects.

MRP8/14 proteins are regarded as markers for a number of inflammatory diseases in humans. Various conditions have shown significant correlation of MRP8/14 levels with disease activity. For example, MRP8/14 levels in stool are a reliable indicator of intestinal inflammatory conditions and can be used to predict relapse in inflammatory bowel diseases. Plasma MRP8/14 level can be a marker for acute rejection in kidney allograft transplantation. Concentrations of MRP8/14 in serum, and particularly in synovial fluid, correlate strongly with disease activity in rheumatoid arthritis. In Systemic Lupus Erythematosus (SLE) patients, serum levels of MRP8/14 are higher than in healthy controls and are associated with disease activity. MRP8/14 may also be a useful biomarker of platelet and inflammatory disease activity in atherothrombosis. Increasing plasma concentrations of MRP-8/14 among healthy individuals can predict the risk of future cardiovascular events.

BioLegend's LEGEND MAX™ Human MRP8/14 (Calprotectin) ELISA Kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with a capture antibody. This kit is specifically designed for the accurate protein quantitation of human MRP8/14 from cell culture supernatant, serum, plasma, and other biological fluids. It is analytically validated with ready-to-use reagents.

Materials Provided:

Description	Quantity	Volume (per bottle)	Part #
Anti-Human MRP8/14 Pre-coated 96-well Strip Microplate	1 plate		79886
Human MRP8/14 Detection Antibody	1 bottle	12 mL	79887
Human MRP8/14 Standard	1 vial	lyophilized	79889
Avidin-HRP B	1 bottle	12 mL	78230
Assay Buffer A	2 bottles	25 mL	78232
Wash Buffer (20X)	1 bottle	50 mL	78233
Substrate Solution D	1 bottle	12 mL	78115
Stop Solution	1 bottle	12 mL	79133
Plate Sealers	4 sheets		78101

Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 µL to 1,000 µL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Plate Shaker
- Paper towels
- Polypropylene vials

Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

<table>
<tr><th colspan="2">Opened or Reconstituted Components</th></tr>
<tr><td>Microplate wells</td><td>If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month.</td></tr>
<tr><td>Standard</td><td>The remaining reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Detection Antibody</td><td rowspan="6">Store opened reagents between 2°C and 8°C and use within one month.</td></tr>
<tr><td>Avidin-HRP B</td></tr>
<tr><td>Assay Buffer A</td></tr>
<tr><td>Wash Buffer (20X)</td></tr>
<tr><td>Substrate Solution D</td></tr>
<tr><td>Stop Solution</td></tr>
</table>

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution F is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with NCCLS regulations.
4. Stop Solution contains strong acid. *Wear eye, hand, and face protection.*
5. Before disposing of the plate, rinse it with an excess amount of tap water.

Tel: 858-768-5800

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Cell Culture Supernatant: If necessary, centrifuge all samples to remove debris prior to analysis. It is recommended that samples be stored at < -70°C. Avoid repeated freeze-thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 x *g*. Remove serum layer and assay immediately or store serum samples at < -70°C. Avoid repeated freeze-thaw cycles.

Plasma: Collect blood samples in citrate, heparin or EDTA containing tubes. Centrifuge for 10 minutes at 1,000 x *g* within 30 minutes of collection. Assay immediately or store plasma samples at < -70°C. Avoid repeated freeze-thaw cycles.

Urine: Collect first-morning, mid-stream urine samples in a sterile container. To remove any possible sediment, centrifuge for 10 minutes at 1,000 x g. Remove supernatant and assay immediately or store at < -70°C. Avoid repeated freeze-thaw cycles.

Saliva: Rinse mouth twice with water 10 minutes before collection. Collect saliva using a sterile microfuge tube or an appropriate saliva collection device. Centrifuge the samples for 10 minutes at 1,000 x g and assay the clear upper layer of the samples immediately or aliquot and store samples at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

Reagent and Sample Preparation:

Note: All reagents should be diluted immediately prior to use.

1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and vortex until dissolved.
2. Reconstitute the lyophilized Human MRP8/14 Standard by adding the volume of Assay Buffer A to make the 200 ng/mL top standard stock solution (Refer toLEGEND MAX Kit Lot-Specific Certificate of Analysis/ LEGEND MAX Kit Protocol). Allow the reconstituted standard to sit at room temperature for 15 minutes, then briefly vortex to mix completely.

3. Serum and plasma samples need to be diluted 1:40 with Assay Buffer A before being assayed. For example, dilute 5 µL of sample with 195 µL of Assay Buffer A.

4. For cell culture supernatant samples, the end user needs to determine the dilution factor in a preliminary experiment. If dilutions are necessary, samples should be diluted with Assay Buffer A.

5. For measuring urine samples, in general, no sample dilution is required. If dilutions are necessary, samples should be diluted with Assay Buffer A.

6. For measuring saliva samples, a 1000-fold dilution using Assay Buffer A is recommended. For example, first dilute 5 µL of sample in 95 µL of Assay Buffer A. Then dilute 5 µL of diluted sample in 245 µL of Assay Buffer A.

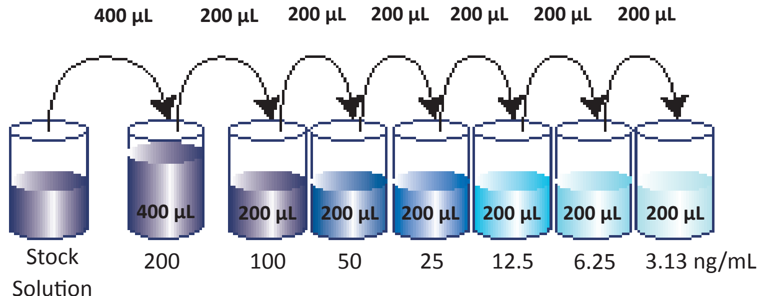
Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.

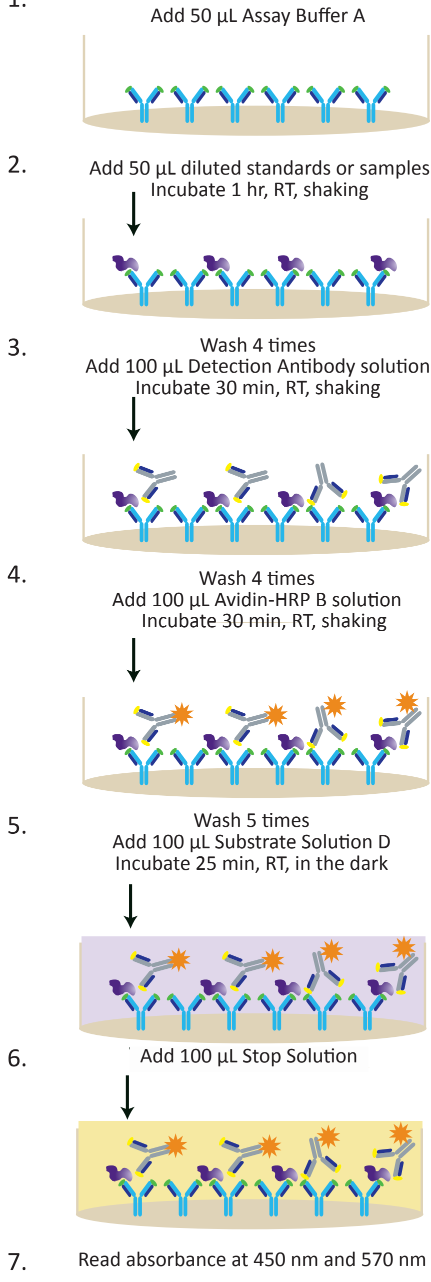
2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Transfer 400 µL of the 200 ng/mL top standard stock solution prepared above to a new tube. Perform six two-fold serial dilutions of the 200 ng/mL top standard in separate tubes using Assay Buffer A as the diluent. Thus, the human MRP8/14 standard concentrations in the tubes are 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 12.5 ng/mL, 6.25 ng/mL and 3.13 ng/mL, respectively. Assay Buffer A serves as the zero standard (0 ng/mL).



4. Add 50 µL of Assay Buffer A to each well that will contain either standard dilutions or samples.
5. Add 50 µL of standard dilutions or samples to the appropriate wells.
6. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 1 hour while shaking at 200 rpm.
7. Discard the contents of the plate into a sink, Wash the plate 4 times with at least 300 µL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes should be performed similarly.
8. Add 100 µL of Human MRP8/14 Detection Antibody solution to each well, seal the plate and incubate at room temperature for 30 min while shaking.
9. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 7.
10. Add 100 µL of Avidin-HRP B solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.
11. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 7. For the final washes, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.
12. Add 100 µL of Substrate Solution D to each well and incubate for 25 minutes in the dark. Wells containing human MRP8/14 should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.
13. Stop the reaction by adding 100 µL of Stop Solution to each well. The solution color should change from blue to yellow.
14. Read absorbance at 450 nm immediately. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

Assay Procedure Summary



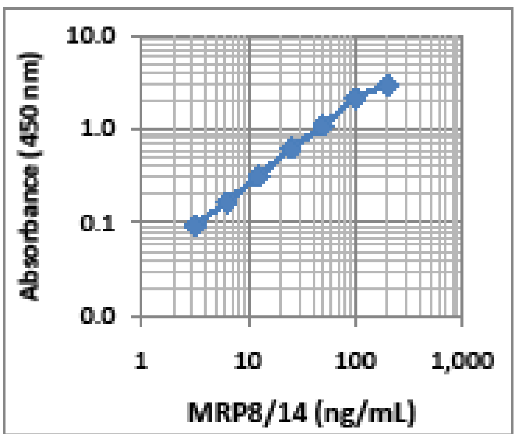
Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with protein concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown protein concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the cytokine concentration.

If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

Typical Data:

This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance Characteristics:

Specificity: No cross-reactivity was observed when this kit was used to analyze 32 human recombinant proteins listed below, each at 50 ng/mL.

Human	MRP8, MRP14, IL-1α, IL-1β, IL-1RA, IL-8, IL-13, IL-17A, IL-34, TNF-α, TNF-β, EGF, IFN-λ1, IFN-β, GROα, Lymphotactin, CCL20, CX3CL1, RANTES,F, ICAM-1, VCAM-1, PDGF-BB, GM-CSF, GARP, sFasL, Lipocalin-2, TWEAK, APRIL, Cystatin C, Nogo B, TIM-1, GAPDH.

Sensitivity: The minimum detectable concentration is 0.62 ± 0.34 ng/mL (n = 5).

Recovery: Recombinant human MRP8/14, at concentrations of 50, 25, and 12.5 ng/mL, was spiked into 10 human serum samples and then analyzed with the LEGEND MAX™ Human MRP8/14 ELISA Kit. On average, 97.1% of the protein was recovered from the serum samples.

Linearity: Nine human serum samples were first diluted 1:40 and then further serially diluted with Assay Buffer A to produce samples with values within the dynamic range and then assayed with the kit. The linearity of dilution ranged from 104.8 to 118.7%. On average, 110.3% linearity of dilution was observed.

Intra-Assay Precision: Two samples with different MRP8/14 concentrations were tested with 16 replicates in one assay.

Concentration	Sample 1	Sample 2
Number of Replicates	16	16
Mean Concentration (ng/mL)	53.9	9.9
Standard Deviation	1.5	0.3
% CV	2.8%	3.0%

Inter-Assay Precision: Two samples with different concentrations of MRP8/14 were assayed in six independent assays.

Concentration	Sample 1	Sample 2
Number of Assays	5	5
Mean Concentration (ng/mL)	45.1	8.1
Standard Deviation	3.4	0.7
% CV	7.5%	8.6%

Biological Samples:

Serum and plasma - Normal human serum (n = 24) and plasma (n = 16) samples were assayed for basal levels of human MRP8/14. Sample levels ranged from 0.11 to 3.52 µg/mL , averaging 1.24 µg/mL.

Cell Culture Supernatant - Human PBMCs were plated at 1x 10^6 /mL in complete RPMI medium and stimulated under various conditions. Supernatants were harvested on day 2 and assayed for human MRP8/14. The result is summarized in the table below:

Stimulation Conditions	**Conc. (µg/mL)**
Unstimulated Control	1.6
CD3 plus CD28 (1 µg/mL each)	2.6
PMA (10 ng/mL) plus Ionomycin (0.5 µg/mL)	3.0

Urine and Saliva - Samples from 3 healthy individuals were tested and the results are summarized below:

Sample	Donor 1	Donor 2	Donor 3
Urine (ng/mL)	159.2	<0.5	3.3
Saliva (µg/mL)	75.3	3.3	117.7

Troubleshooting Guide:

Problem	Probable Cause	Solution
High Background	Background wells were contaminated	Avoid cross-well contamination by using the provided plate sealers. Use multichannel pipettes and change tips between pipetting samples and reagents.
	Insufficient washes	Increase number of washes. Increase soaking time between washes prior to addition of substrate solution.
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
No or poor signal	Detection Antibody, Avidin-HRP or Substrate solution were NOT added	Rerun the assay and follow the protocol.
	Wrong reagent or reagents were added in wrong sequential order	
	Insufficient plate agitation	The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing.
	The wash buffer contains Sodium Azide (NaN3)	Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity.
	Incubations were done at an inappropriate temperature, timing or without agitation	Rerun the assay and follow the protocol.
Low or poor standard curve signal	The standard was incorrectly reconstituted or diluted	Adjust the calculations and follow the protocol.
	Standard was inappropriately stored	Store the reconstituted standard stock solution in polypropylene vials at -70°C. Avoid repeated freeze-thaw cycles.
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and the correct reagent volume.

Problem	Probable Cause	Solution
Signal is high, standard curves have saturated signal	Standard reconstituted with less volume than required	Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol.
	Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long	Rerun the assay and follow the protocol.
Sample readings are out of range	Samples contain no or below detectable levels of the analyte	If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications.
	Samples contain analyte concentrations greater than highest standard point	Samples may require dilution and analysis.
High variation in samples and/or standards	Multichannel pipette errors	Confirm that pipette calibrations are accurate.
	Plate washing was not adequate or uniform	Ensure pipette tips are tightly secured. Ensure uniformity in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before assaying.
	Samples may have high particulate matter	Remove particulate matter by centrifugation.
	Cross-well contamination	Do not reuse plate sealers. Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.

Tel: 858-768-5800

ELISA Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

LEGEND MAX™ Kits are manufactured by **BioLegend Inc.**
8999 BioLegend Way
San Diego, CA 92121
Tel: 1.858.768.5800
Tel US & Canada Toll-Free: 1.877.Bio-Legend (1.877.246.5343)
Fax: 1.877.455.9587
Email: info@biolegend.com
biolegend.com

439707-R06