Quantikine® ELISA

Human S100A8/S100A9 Heterodimer Immunoassay

Catalog Number DS8900

For the quantitative determination of human S100A8/S100A9 Heterodimer concentrations in cell culture supernates, tissue lysates, serum, plasma, saliva, urine, human milk, and fecal extract.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

S100A8 (also known as MRP8, Calgranulin A, and CP-10) and S100A9 (also known as MRP14 and Calgranulin B) are pro-inflammatory members of the S100 family of secreted calcium binding proteins (1, 2). The 10 kDa human S100A8 and 14 kDa S100A9 each contain two EF-hand calcium binding motifs. Human S100A8 shares 57% and 61% amino acid (aa) sequence identity with mouse and rat S100A8, respectively. Human S100A9 shares 57% and 62% amino acid sequence identity with mouse and rat S100A9, respectively (3, 4). In the presence of calcium or zinc, S100A8 and S100A9 can associate into disulfide-linked homodimers and 34-35 kDa heterodimers known as S100A8/S100A9 Heterodimer or Calprotectin (5-8).

S100A8 and S100A9 are upregulated in neutrophils, monocytes, Schwann cells, and keratinocytes at sites of inflammation (9-15). The S100A8/S100A9 Heterodimer is elevated in rheumatoid arthritis synovial fluid and in the serum of cardiovascular disease, atopic dermatitis, and psoriatic arthritis patients (1, 11, 15-17). Its levels correlate with body adiposity and leukocyte count (18). S100A8/S100A9 Heterodimer exerts its effects through the receptors RAGE and TLR4 (13, 19). The heterodimer promotes neutrophil infiltration into sites of inflammation and inflammatory cytokine production by monocytes (6, 10, 11, 14, 20). It promotes bone spur formation in osteoarthritis (21), upregulation of Complement Component C3 (20), astrocyte proliferation (19), and the suppression of tumor growth by promoting the influx and activation of NK cells (12, 13). The S100A8/S100A9 Heterodimer additionally binds to fatty acids such as arachidonic acid (9), and it sequesters manganese, thereby restricting the growth of Mn-dependent bacteria (22).

The Quantikine Human S100A8/S100A9 Heterodimer Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human S100A8/S100A9 Heterodimer in cell culture supernates, tissue lysastes, serum, plasma, saliva, urine, human milk, and fecal extract. It contains *E. coli*-expressed recombinant human S100A8/S100A9 Heterodimer and antibodies raised against the recombinant factor. Results obtained using natural human S100A8/S100A9 Heterodimer showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human S100A8/S100A9 Heterodimer.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human S100A8/S100A9 Heterodimer has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any S100A8/S100A9 Heterodimer present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human S100A8/S100A9 Heterodimer is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of S100A8/S100A9 Heterodimer bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human S100A8/S100A9 Heterodimer Microplate	898152	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human S100A8/ S100A9 Heterodimer.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human S100A8/S100A9 Heterodimer Standard	898154	2 vials of recombinant human S100A8/ S100A9 Heterodimer in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard for each assay.
Human S100A8/S100A9 Heterodimer Conjugate	898153	21 mL of a monoclonal antibody specific for human S100A8/S100A9 Heterodimer conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-34	895265	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-10	895266	3 vials (21 mL/vial) of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human S100A8/S100A9 Heterodimer Controls (optional; R&D Systems, Catalog # QC223).

SUPPLIES REQUIRED FOR TISSUE LYSATE SAMPLES

- RIPA buffer with protease inhibitors

PRECAUTIONS

S100A8/S100A9 Heterodimer is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Tissue Lysates - Tissue samples were lysed prior to assay as described in the Sample Values section.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Fecal Extract - Fecal samples were prepared prior to assay as described in the Sample Values section.

SAMPLE PREPARATION

Serum samples require a 200-fold dilution due to high endogenous levels. A suggested 200-fold dilution can be achieved by adding 20 µL of sample to 180 µL of Calibrator Diluent RD5-10. Complete the 200-fold dilution by adding 10 µL of the diluted sample to 190 µL Calibrator Diluent RD5-10.

Plasma samples require a 100-fold dilution due to high endogenous levels. A suggested 100-fold dilution is 10 µL of sample + 990 µL of Calibrator Diluent RD5-10.

Saliva samples require a 500-fold dilution due to high endogenous levels. A suggested 500-fold dilution can be achieved by adding 10 µL of sample to 490 µL of Calibrator Diluent RD5-10. Complete the 500-fold dilution by adding 20 µL of the diluted sample to 180 µL Calibrator Diluent RD5-10.

Human milk samples require at least a 10-fold dilution due to high endogenous levels. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5-10.

For tissue lysate and fecal extract samples, quantitation of sample protein concentration using a total protein assay is recommended. The suggested range for total tissue lysate protein added is 1-5 µg/well. The suggested range for total fecal extract protein added is 5-50 µg/well.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

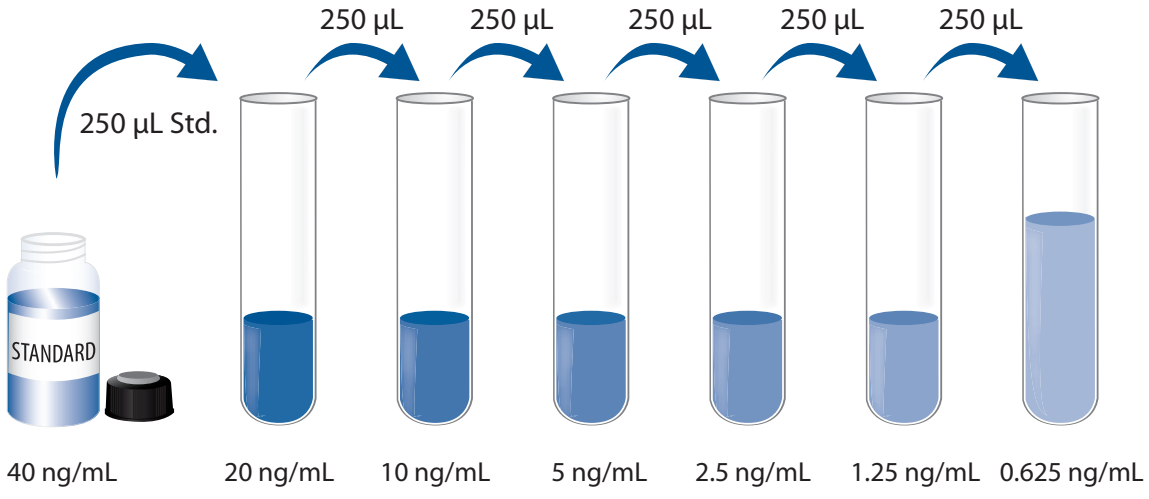
Note: *S100A8/S100A9 Heterodimer is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Human S100A8/S100A9 Heterodimer Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human S100A8/S100A9 Heterodimer Standard with Calibrator Diluent RD5-10. This reconstitution produces a stock solution of 40 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 250 µL of Calibrator Diluent RD5-10 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human S100A8/S100A9 Heterodimer Standard (40 ng/mL) serves as the high standard. Calibrator Diluent RD5-10 serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *S100A8/S100A9 Heterodimer is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-34 to each well.
4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human S100A8/S100A9 Heterodimer Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

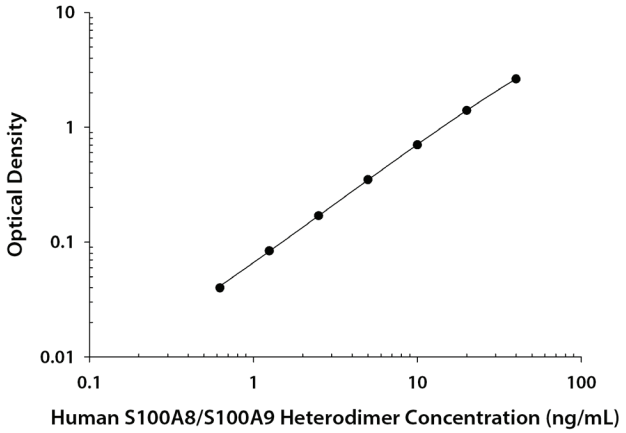
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human S100A8/S100A9 Heterodimer concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.004 0.005	0.005	—
0.625	0.044 0.045	0.045	0.040
1.25	0.087 0.091	0.089	0.084
2.5	0.173 0.176	0.175	0.170
5	0.355 0.357	0.356	0.351
10	0.698 0.720	0.709	0.704
20	1.381 1.434	1.408	1.403
40	2.587 2.696	2.642	2.637

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (ng/mL)	5.64	12.1	25.3	6.09	12.6	24.6
Standard deviation	0.155	0.370	1.14	0.352	0.606	0.792
CV (%)	2.7	3.1	4.5	5.8	4.8	3.2

RECOVERY

The recovery of human S100A8/S100A9 Heterodimer spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	85	79-96%
Lysis buffer* (n=3)	94	80-104%
Urine* (n=4)	100	91-113%
Human milk* (n=4)	96	83-103%

*Samples were diluted prior to assay.

SENSITIVITY

Twenty-one assays were evaluated and the minimum detectable dose (MDD) of human S100A8/S100A9 Heterodimer ranged from 0.005-0.215 ng/mL. The mean MDD was 0.086 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against highly purified *E. coli*-expressed recombinant human S100A8/S100A9 Heterodimer produced at R&D Systems.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human S100A8/S100A9 Heterodimer were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Lysis buffer (n=3)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	104	107	104	106	104
	Range (%)	100-112	99-119	99-109	102-109	101-109
1:4	Average % of Expected	104	110	110	111	110
	Range (%)	99-112	100-120	106-116	102-115	99-119
1:8	Average % of Expected	103	112	111	111	116
	Range (%)	101-105	105-121	107-118	100-117	101-124
1:16	Average % of Expected	99	114	113	104	115
	Range (%)	95-104	103-125	106-116	101-109	101-124

		Saliva* (n=4)	Urine* (n=4)	Human milk* (n=4)	Fecal extract* (n=3)
1:2	Average % of Expected	103	102	103	103
	Range (%)	100-107	101-105	101-107	96-112
1:4	Average % of Expected	105	104	104	103
	Range (%)	102-115	101-109	99-108	94-113
1:8	Average % of Expected	105	105	102	105
	Range (%)	101-115	97-118	99-105	94-111
1:16	Average % of Expected	101	102	100	88
	Range (%)	92-112	87-118	90-105	86-90

*Samples were diluted prior to assay.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human S100A8/S100A9 Heterodimer in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=72)	2015	481-6540	1219
EDTA plasma (n=40)	473	127-1395	295
Heparin plasma (n=40)	830	298-1640	314
Saliva (n=10)	7271	2137-18,960	5336
Urine (n=10)	102	0.858-359	99.7
Human milk (n=10)	1308	34.0-5720	1876

Cell Culture Supernates:
Human peripheral blood leukocytes (PBL; $1x10^6$) were cultured in RPMI 1640 and supplemented with 10% fetal bovine serum. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 or 5 days. Aliquots of the cell culture supernates were removed and assayed for levels of human S100A8/S100A9 Heterodimer.

Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Unstimulated	399	575
Stimulated	588	1091

THP-1 human acute monocytic leukemia cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cells were cultured unstimulated or stimulated with 200 nM PMA until confluent. An aliquot of the cell culture supernate was removed, assayed for human S100A8/S100A9 Heterodimer, and measured 11.6 ng/mL and 38.0 ng/mL, respectively.

Tissue Lysates - Human colon tissue samples were prepared in RIPA buffer with protease inhibitors. The working buffer was kept on ice and used within 1 day. Aliquots of the tissue lysates were removed and assayed for human S100A8/S100A9 Heterodimer.

Sample Type	(ng/mL)
Human colon, normal	14,700
Human colon, cancer	11,215

Fecal Extracts - Three fecal samples were extracted by adding 5 mL extraction buffer (0.1 M Tris, 0.015 M NaCl, 1.0 M Urea, 1.0 mM $CaCl_2$, 0.1 M Citric Acid Monohydrate, 5 mg/mL BSA, and 0.25% Gentamycin Sulfate at pH 8.0) into 100 mg sample. Samples were vortexed for 10 minutes and filtered through a 0.2 µm filter. Samples were assayed for human S100A8/S100A9 Heterodimer and measured 81.2 ng/mL, 8.71 ng/mL, and 1175 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human S100A8/S100A9 Heterodimer.

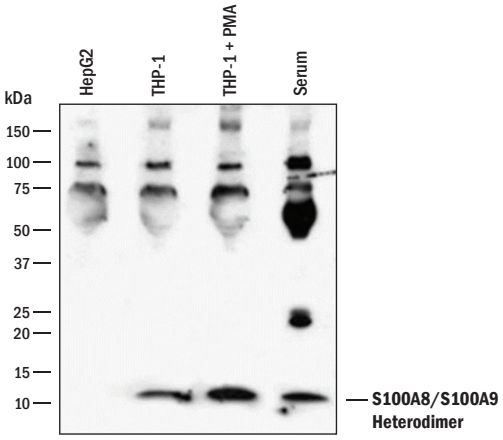
The factors listed below were prepared at 500 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 500 ng/mL in a mid-range control were assayed for interference. No significant cross-reactivity or interference was observed.

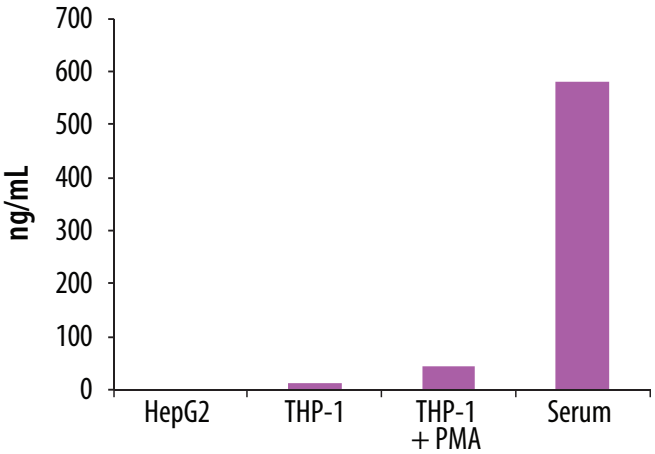
Recombinant human:

RAGE
S100A1
S100A2
S100A4
S100A6
S100A7
S100A8
S100A9
S100A10
S100A11
S100A13
S100A16
S100B
S100P
TLR-4
TLR-4/MD-2 Complex

Recombinant mouse:

S100A8
S100A9
S100A8/S100A9 Heterodimer





Human serum and conditioned media were analyzed by Western blot and Quantikine ELISA. THP-1 human acute monocytic leukemia cells were left unstimulated or treated with PMA for 24 hours prior to harvest, and HepG2 human hepatocellular carcinoma cells were left unstimulated. For the Western blot, serum was diluted 1:100, while conditioned medias were run neat. Samples were resolved under reducing SDS-PAGE conditions, transferred to PVDF membrane, and immunoblotted with sheep anti-human S100A8 (R&D Systems, Catalog # AF4570). The Western blot shows a direct correlation with the ELISA value for these samples.

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