Quantikine® ELISA

Mouse RAGE Immunoassay

Catalog Number MRG00

For the quantitative determination of mouse Receptor for Advanced Glycation End product (RAGE) concentrations in cell culture supernates, tissue lysates, serum, plasma, and urine.

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

Receptor for Advanced Glycation End product (RAGE) is an approximately 50 kDa type I transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily. It binds a variety of ligands including Advanced Glycation End products (AGEs), beta-amyloid peptides (Aβ), HMGB1/Amphoterin, and several S100 family proteins. AGEs are adducts formed by the non-enzymatic glycation and oxidation of proteins and lipids. This process occurs during the normal course of aging and is dramatically accelerated in diabetes where hyperglycemia is a major trigger. RAGE plays important roles in several pathological processes including inflammation, diabetes, cancer, and Alzheimer's disease (AD) (1-3). RAGE consists of a 319 amino acid (aa) extracellular domain (ECD) with three Ig-like domains, a 21 aa transmembrane segment, and a 41 aa cytoplasmic domain (4). Within the ECD, mouse RAGE shares 79% and 91% aa sequence identity with human and rat RAGE, respectively. Alternative splicing of mouse RAGE generates multiple additional isoforms that are truncated at various points within the ECD or carry internal deletions (5). A soluble form of RAGE (sRAGE) can also be generated by metalloproteinase-mediated cleavage of the ECD (6). The membrane-bound fragment remaining after ECD shedding can be cleaved by gamma-secretase to release the intracellular domain (6).

RAGE is expressed in the central nervous system (CNS) during development as well as in adult endothelial cells, smooth muscle cells, pericytes, monocytes, and neurons (7, 8). RAGE contributes to the severity of diseases with an inflammatory component. It is locally upregulated in vascular inflammation (e.g. diabetes, atherosclerosis, vascular injury) (9-11). At these sites, RAGE binding to S100A1, EN-RAGE/S100A12, or S100B induces inflammatory immune cell adhesion and infiltration (9, 12, 13) as well as vascular smooth muscle proliferation, neointimal expansion, and atherosclerotic plaque development (10, 11, 14). RAGE also cooperates with TLR9 in the B cell and dendritic cell inflammatory response to complexes of HMGB1 and CpG DNA (15). In cancer, RAGE binding to HMGB1, S100A8, or S100A9 promotes tumor growth and metastasis in addition to inflammatory cell infiltration (16, 17). sRAGE functions as a sink for RAGE ligands and lessens the severity of these processes (9, 16, 18-20). Serum levels of sRAGE are decreased in patients with coronary artery disease (21). The system is positively regulated by RAGE activation which promotes the upregulation of S100A8 and S100A9 and negatively regulated by S100A8/A9 heterodimers which promote the release of sRAGE (17, 22).

In the nervous system, RAGE binds to both the 1-40 and 1-42 forms of Aβ, leading to increased inflammation, oxidative stress, and cytotoxicity (23-25). It is upregulated in Alzheimer's disease neurons, microglia, and vasculature (23). RAGE is also upregulated on endothelial cells of the blood-brain barrier (BBB), where it mediates the transport of Aβ into the cerebrospinal fluid (CSF) (24). sRAGE is not transported across the BBB but can be locally released into the CSF (24, 26). Its circulating levels are decreased in Alzheimer's disease, enabling the enhanced transport of Aβ across the BBB as well as Aβ-induced brain inflammation (24, 27). RAGE-S100 protein interactions contribute to disease progression in the EAE model of multiple sclerosis (28).

The Quantikine® Mouse RAGE Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse RAGE levels in cell culture supernates, tissue lysates, serum, plasma, and urine. It contains NS0-expressed recombinant mouse RAGE and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant mouse RAGE accurately. Results obtained using natural mouse RAGE showed dose-response 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 natural mouse RAGE.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse RAGE has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any RAGE present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse RAGE is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of RAGE bound in the initial step. The sample values are then read off the standard curve.

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 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.
- 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.

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
Mouse RAGE Microplate	893190	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse RAGE.	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.*
Mouse RAGE Conjugate	893191	12 mL of a polyclonal antibody specific for mouse RAGE conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse RAGE Standard	893192	Recombinant mouse RAGE in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Mouse RAGE Control	893193	Recombinant mouse RAGE in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Assay Diluent RD1W	895038	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5P	895151	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
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	895174	23 mL of diluted hydrochloric 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.
- 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 rpm ± 50 rpm.
- **Polypropylene** test tubes for dilution of standards and samples.

SUPPLIES REQUIRED FOR TISSUE LYSATE SAMPLES

- Cell Lysis Buffer 2 (R&D Systems®, Catalog # 895347).
- PBS

PRECAUTIONS

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. 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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Tissue Lysates - Tissues must be lysed prior to assay as described in the Sample Values section.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Variations in sample collection, processing, and storage may cause sample value differences. Adhere to consistent clotting times.*

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 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.*
Hemolyzed samples are not suitable for use in this assay.

Urine - Collect urine using a metabolic cage. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Centrifuge again before assaying to remove any additional precipitates that may appear after storage.

SAMPLE PREPARATION

Serum and plasma samples require a 10-fold dilution. A suggested 10-fold dilution is 15 µL of sample + 135 µL of Calibrator Diluent RD5P (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse RAGE Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

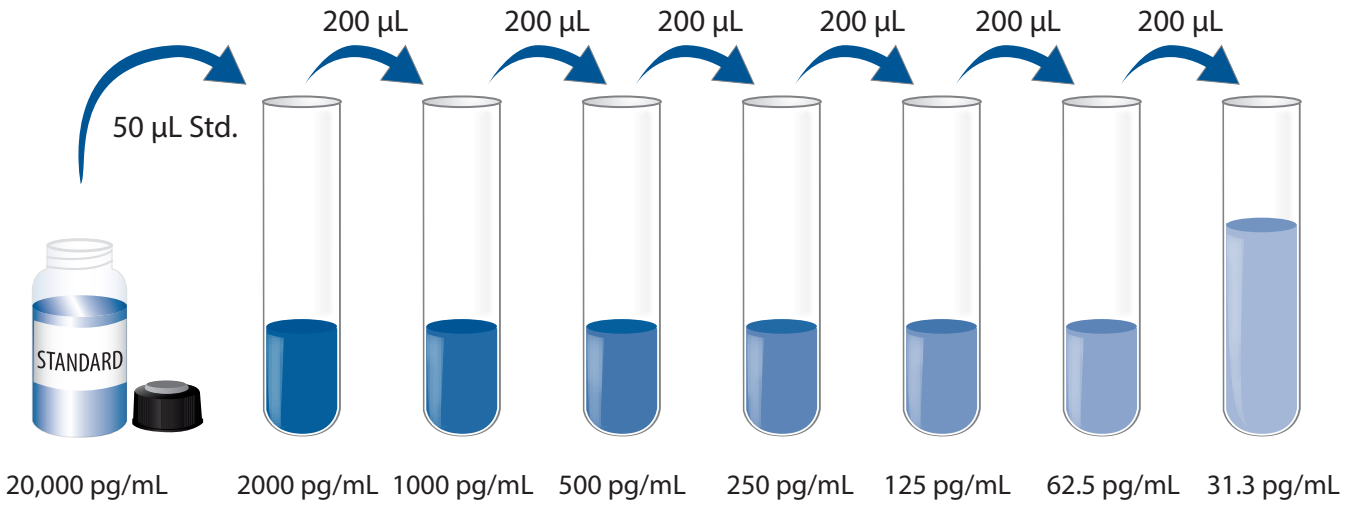
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Calibrator Diluent RD5P (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5P to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5P (diluted 1:5).

Mouse RAGE Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse RAGE Standard with deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle mixing prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5P (diluted 1:5) into the 2000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2000 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

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

1. Prepare all reagents, standards, control, and samples as directed by 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 RD1W 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. A plate layout is provided to record standards and samples assayed.
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 100 µL of Mouse RAGE 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 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 100 µL of Stop Solution to each well. 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 Sample Preparation section.

CALCULATION OF RESULTS

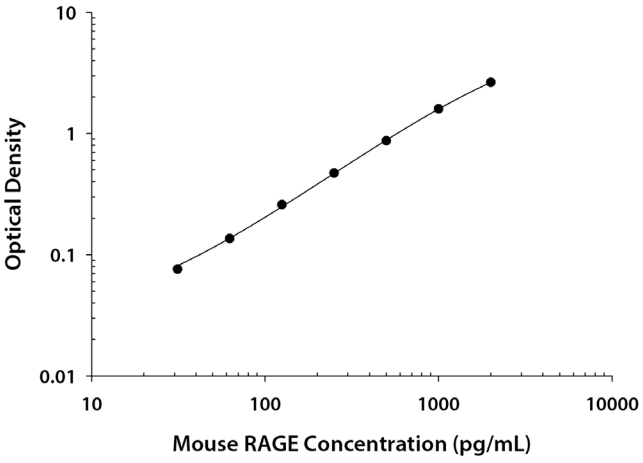
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 mouse RAGE 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.



(pg/mL)	O.D.	Average	Corrected
0	0.025 0.029	0.027	—
31.3	0.103 0.103	0.103	0.076
62.5	0.160 0.165	0.163	0.136
125	0.282 0.289	0.286	0.259
250	0.486 0.512	0.499	0.472
500	0.889 0.907	0.898	0.871
1000	1.620 1.630	1.625	1.598
2000	2.613 2.719	2.666	2.639

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 (pg/mL)	66.1	236	518	68.8	242	559
Standard deviation	5.02	14.5	24.3	5.44	13.4	32.6
CV (%)	7.6	6.1	4.7	7.9	5.5	5.8

RECOVERY

The recovery of mouse RAGE spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture samples (n=4)	108	94-120%
Tissue lysates (n=4)*	103	91-120%
Serum (n=4)*	107	90-117%
EDTA plasma (n=4)*	101	87-118%
Heparin plasma (n=4)*	111	96-120%
Urine (n=4)*	109	96-120%

*Samples were diluted prior to assay.

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of mouse RAGE were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

		Cell culture supernates (n=4)	Tissue lysates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	92	103	101	102	99	99
	Range (%)	89-94	98-107	94-111	100-103	98-101	94-104
1:4	Average % of Expected	92	103	100	104	100	99
	Range (%)	88-94	98-106	93-111	101-106	99-102	89-106
1:8	Average % of Expected	92	101	102	107	103	100
	Range (%)	87-95	94-113	98-114	102-117	100-105	85-110
1:16	Average % of Expected	90	102	102	102	102	97
	Range (%)	85-95	92-120	92-117	93-110	88-109	86-104

SENSITIVITY

Thirty-five assays were evaluated and the minimum detectable dose (MDD) of mouse RAGE ranged from 0.900-4.81 pg/mL. The mean MDD was 2.36 pg/mL.

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

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant mouse RAGE produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Urine - Samples were evaluated for the presence of mouse RAGE in this assay.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=10)	4851	743-14,313	5150
EDTA plasma (n=5)	3184	1102-5676	2256
Heparin plasma (n=5)	2460	1314-5425	1696
Urine (n=10)	1302	201-2206	722

Cell Culture Supernates - Organs from mice were rinsed with enough PBS to cover the organs and kept on ice. Tissue was homogenized with a tissue homogenizer and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 3 days. Aliquots of the cell culture supernates were removed and assayed for levels of mouse RAGE.

Tissue	Observed Value (pg/mL)
Liver	2210
Lung	1,289,028
Spleen	67.1

Tissue Lysates - Organs from mice were rinsed with enough PBS to cover the organs, cut into 1-2 mm pieces, and homogenized with a tissue homogenizer in PBS. An equal volume of Cell Lysis Buffer 2 was added and tissues were lysed at room temperature for 30 minutes with gentle agitation. Debris was then removed by centrifugation. Aliquots of the lysates were removed and assayed for levels of mouse RAGE.

Tissue	Observed Value (pg/mL)
Brain	5968
Liver	3131
Lung	33,330,000
Spleen	2844

SPECIFICITY

This assay recognizes natural and recombinant mouse RAGE.

The factors listed below were prepared at 100 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a mid-range recombinant mouse RAGE control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:
CD36/SR-B3
Galectin-3
LOX-1/OLR1
S100A10
SR-B1
Stabilin-2

Recombinant human:
Amyloid β-Peptide
EN-RAGE
HMGB1
S100B

Recombinant canine:
RAGE

Natural proteins:
bovine AGE-BSA

Recombinant rat RAGE cross-reacts approximately 24% in this assay.

Recombinant human RAGE cross-reacts approximately 9% in this assay.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.