Quantikine® ELISA

Human RAGE Immunoassay

Catalog Number DRG00
SRG00
PDRG00

For the quantitative determination of the extracellular domain of human Receptor for Advanced Glycation End product (RAGE) concentrations in cell culture supernates, serum, and plasma.

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 (1-3). 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). RAGE consists of a 320 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, human RAGE shares 79% aa sequence identity with mouse and rat RAGE. Alternative splicing of human RAGE generates multiple additional isoforms that are truncated at various points within the ECD or carry internal deletions (5-7). A soluble form of RAGE (sRAGE) can also be generated by metalloproteinase-mediated cleavage of the ECD (8). The membrane-bound fragment remaining after ECD shedding can be cleaved by gamma-secretase to release the intracellular domain (8).

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 (9, 10). RAGE contributes to the severity of diseases with an inflammatory component. It is locally upregulated in vascular inflammation (e.g. diabetes, atherosclerosis, vascular injury) (11-13). At these sites, RAGE binding to S100A1, EN-RAGE/S100A12, or S100B induces inflammatory immune cell adhesion and infiltration (11, 14, 15) as well as vascular smooth muscle proliferation, neointimal expansion, and atherosclerotic plaque development (12, 13, 15, 16). RAGE also cooperates with TLR9 in the B cell and dendritic cell inflammatory response to complexes of HMGB1 and CpG DNA (17). In cancer, RAGE binding to HMGB1, S100A8, or S100A9 promotes tumor growth and metastasis in addition to inflammatory cell infiltration (18, 19). sRAGE functions as a sink for RAGE ligands and lessens the severity of these processes (11, 18, 20-22). Serum levels of sRAGE are decreased in patients with coronary artery disease (23). 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 (19, 24).

In the nervous system, RAGE binds to both the 1-40 aa and 1-42 aa forms of Aβ, leading to increased inflammation, oxidative stress, and cytotoxicity (25-27). It is upregulated in Alzheimer's disease neurons, microglia, and vasculature (25). 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) (26). sRAGE is not transported across the BBB but can be locally released into the CSF (26, 28). 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 (26, 29). In RAGE-S100 protein interactions contribute to disease progression in the EAE model of multiple sclerosis (30).

The Quantikine® Human RAGE Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human RAGE (extracellular domain) in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant human RAGE/Fc Chimera and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human RAGE 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 natural human RAGE.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human RAGE (extracellular domain) has been pre-coated onto a microplate. Standards 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 human RAGE (extracellular domain) 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 RAGE 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, dilute the samples with the appropriate 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.
- 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 #	CATALOG # DRG00	CATALOG # SRG00	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human RAGE Microplate	892603	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human 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.*
Human RAGE Conjugate	892604	1 vial	6 vials	21 mL/vial of a polyclonal antibody specific for human RAGE conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human RAGE Standard	892605	1 vial	6 vials	Recombinant human RAGE in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-60	895328	1 vial	6 vials	11 mL/vial of a buffered protein base with preservatives and blue dye.	
Calibrator Diluent RD5-5	895485	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives. *For cell culture supernate samples.*	
Calibrator Diluent RD6-10	895468	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives *For serum/plasma samples.*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

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

DRG00 contains sufficient materials to run an ELISA on one 96 well plate.
SRG00 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PDRG00). Specific vial counts of each component may vary. Refer to the PharmPak Contents section for specific vial counts.

PHARMPAK CONTENTS

Each PharmPak contains reagents sufficient for the assay of 50 microplates (96 wells/plate). The package inserts supplied are the same as those supplied in the single kit packs and because of this, a few minor differences related to the number of reagents and their container sizes should be noted.

- Sufficient material is supplied to perform at least 50 standard curves; reuse of each vial may be required. The number of vials, and the number of standard curves obtained per vial will vary with the analyte.
- Wash Buffer 25X Concentrate is bulk packed in 125 mL bottles containing 100 mL.
  Note: *Additional wash buffer is available for purchase (R&D Systems®, Catalog # WA126).*

The reagents provided in this PharmPak are detailed below.

PART	PART #	QUANTITY
Human RAGE Microplate	892603	50 plates
Human RAGE Conjugate	892604	50 vials
Human RAGE Standard	892605	25 vials
Calibrator Diluent RD5-5	895485	50 vials
or		
Calibrator Diluent RD6-10	895468	50 vials
Assay Diluent RD1-60	895328	50 vials
Color Reagent A	895000	50 vials
Color Reagent B	895001	50 vials
Wash Buffer Concentrate, 25X	895126	9 bottles
Stop Solution	895032	50 vials
Plate Sealers	N/A	100 sheets
Package Inserts	751067	2 booklets

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.
- Test tubes for dilution of standards.
- Human RAGE Controls (optional; R&D Systems®, Catalog # QC152).

PRECAUTIONS

Calibrator Diluent RD6-10 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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 SDS 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.

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.*

REAGENT PREPARATION

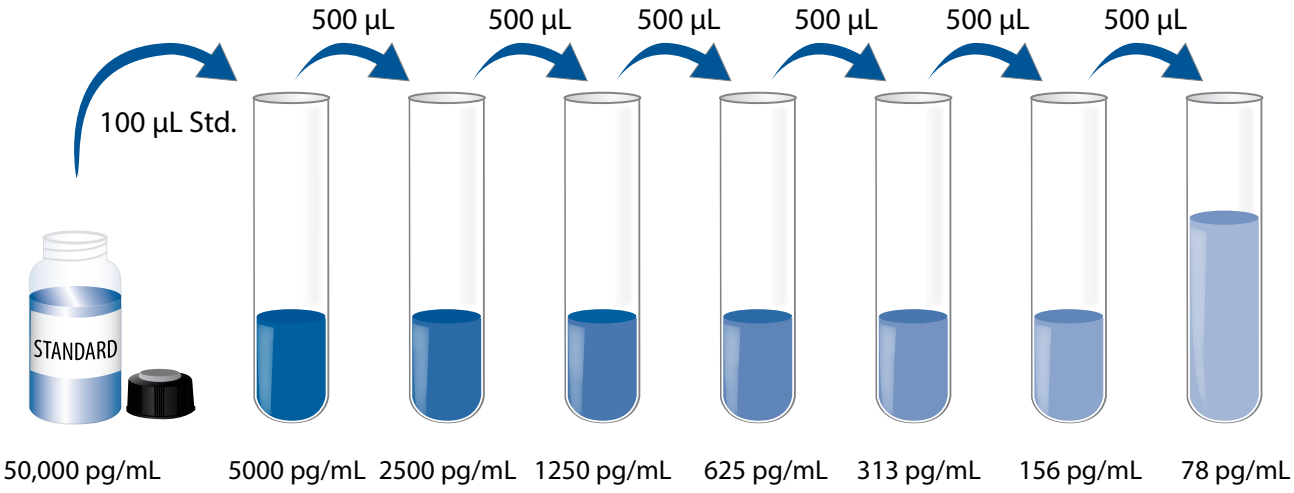
Bring all reagents to room temperature before use.

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 480 mL of 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 RAGE Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human RAGE Standard with deionized or distilled water. This reconstitution produces a stock solution of 50,000 pg/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 900 µL of Calibrator Diluent RD5-5 (*for cell culture supernate samples*) or Calibrator Diluent RD6-10 (*for serum/plasma samples*) into the 5000 pg/mL tube. Pipette 500 µL of the appropriate calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 5000 pg/mL standard serves as the high standard. The appropriate calibrator diluent 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, controls, and samples be assayed in duplicate.

1. Prepare all reagents and working standards 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 100 µL of Assay Diluent RD1-60 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.
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 RAGE Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
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. **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.

CALCULATION OF RESULTS

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

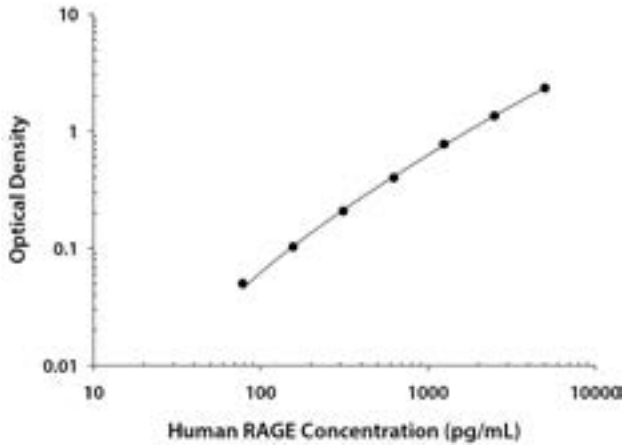
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human 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

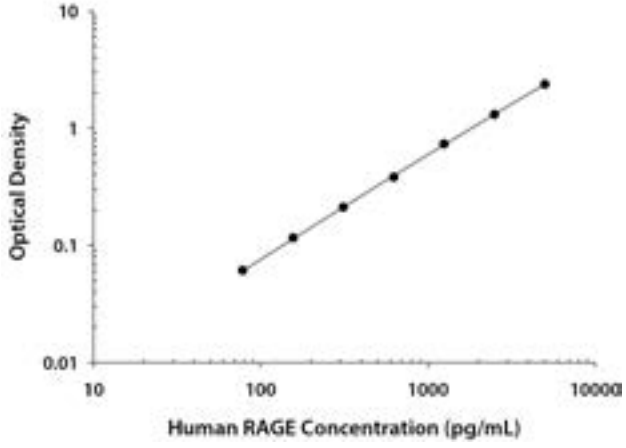
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.010 0.011	0.011	—
78	0.059 0.063	0.061	0.050
156	0.112 0.117	0.114	0.103
313	0.214 0.224	0.219	0.208
625	0.398 0.422	0.410	0.399
1250	0.783 0.791	0.787	0.776
2500	1.360 1.360	1.360	1.349
5000	2.280 2.410	2.350	2.339

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.009 0.009	0.009	—
78	0.069 0.071	0.070	0.061
156	0.123 0.128	0.125	0.116
313	0.216 0.227	0.221	0.212
625	0.372 0.408	0.390	0.381
1250	0.698 0.789	0.743	0.734
2500	1.290 1.360	1.320	1.311
5000	2.340 2.440	2.390	2.381

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 forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	571	1549	3189	563	1568	3159
Standard deviation	34	73	191	49	110	193
CV (%)	6.0	4.7	6.0	8.7	7.0	6.1

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	546	1527	3117	519	1449	2890
Standard deviation	34	73	189	43	119	192
CV (%)	6.2	4.8	6.1	8.3	8.2	6.6

RECOVERY

The recovery of human RAGE spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	98	86-108%

SENSITIVITY

Fifty-seven assays were evaluated and the minimum detectable dose (MDD) of human RAGE ranged from 1.23-16.14 pg/mL. The mean MDD was 4.12 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.

LINEARITY

To assess linearity of the assay, samples containing and/or spiked with high concentrations of human RAGE were diluted with the appropriate calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=3)	EDTA plasma (n=3)	Heparin plasma (n=3)
1:2	Average % of Expected	102	102	103	103
	Range (%)	99-103	99-109	100-110	100-105
1:4	Average % of Expected	102	101	106	108
	Range (%)	99-105	93-112	102-109	106-109
1:8	Average % of Expected	101	104	106	109
	Range (%)	93-109	101-110	101-112	107-110
1:16	Average % of Expected	95	110	112	113
	Range (%)	90-99	104-115	111-113	107-120

CALIBRATION

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

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human RAGE in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=42)	1794	368-4354	755
EDTA plasma (n=42)	1655	382-4329	693
Heparin plasma (n=41)	1908	369-4460	700

Cell Culture Supernates:
Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed and assayed for levels of human RAGE. No detectable levels of human RAGE were observed.

JE-3 human epithelial choriocarcinoma cells were cultured in DMEM with 10% fetal bovine serum. An aliquot of the cell culture supernate was removed, assayed for human RAGE, and measured 648 pg/mL.

SPECIFICITY

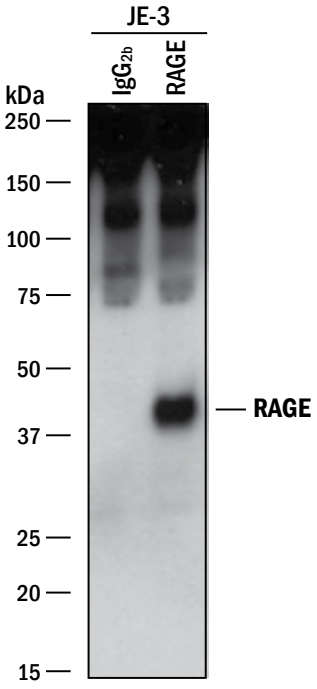
This assay recognizes natural and recombinant human RAGE.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human RAGE control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:
EN-RAGE
HMG-1
S100A10
S100B

Natural proteins:
bovine AGE-BSA

Recombinant mouse and rat RAGE cross-react approximately 2% in this assay.



Conditioned media from human JE-3 cells were analyzed by Immunoprecipitation/Western Blot and Quantikine® ELISA. For immunoprecipitation, samples were diluted in DYC002, and incubated with 2 µg of the capture antibody used in this kit plus 40 µL of Protein G Sepharose beads overnight. Immunoprecipitated samples were resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with the conjugate antibody used in this kit. The kit capture antibody immunprecipitates a band from the ELISA positive sample that is recognized by the kit detection antibody used in the Western Blot. The Quantikine® ELISA value was 2377 pg/mL.

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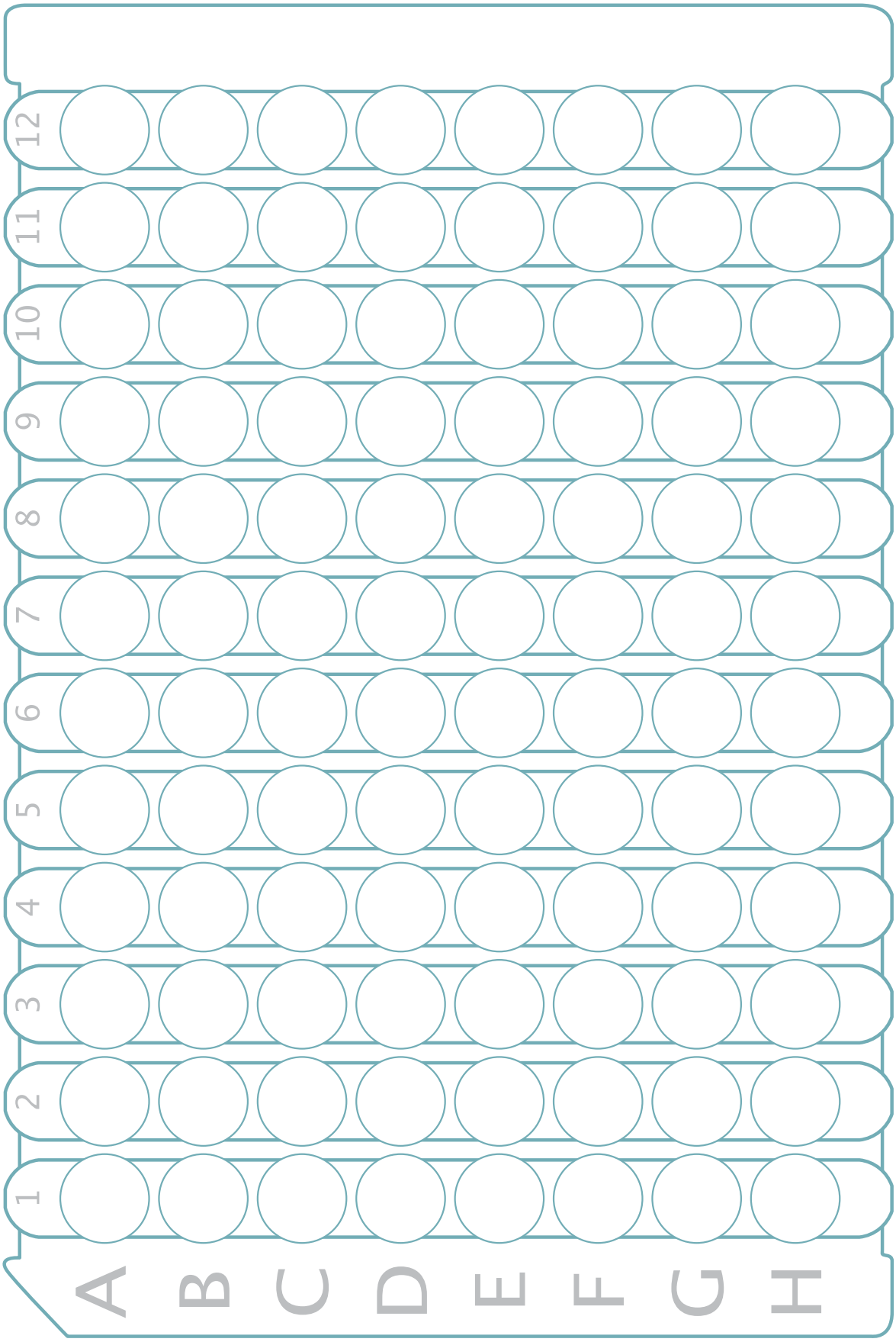
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES