Quantikine®

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.

TABLE OF CONTENTS

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INTRODUCTION

Receptor for Advanced Glycation End product (RAGE) is a multi-ligand type I transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily (1, 2). RAGE has potential involvement in several pathological processes including diabetes, Alzheimer's disease (AD), systemic amyloidosis, and tumor growth (2). RAGE may also mediate physiological functions, such as neuronal outgrowth, survival, and regeneration, and play a part in pro-inflammatory reactions (3 - 5). RAGE is highly expressed during development, especially in the central nervous system (CNS) (6). It is also expressed at lower levels in adult cells including endothelial and smooth muscle cells, mononuclear phagocytes, pericytes, neurons, cardiac myocytes, and hepatocytes (7). RAGE ligands include advanced glycation end products (AGEs), amyloid-β (Aβ) peptide, HMG-1 (also known as Amphoterin), and several members of the S100 protein superfamily (5, 6, 8, 9). Naturally occurring soluble RAGE (sRAGE) splice variants have been identified and could potentially act as endogenous inhibitors of RAGE activity (10 - 12).

RAGE was originally characterized based on its ability to bind AGEs, adducts formed by the non-enzymatic glycation and oxidation of proteins and lipids (13, 14). This process occurs during the normal course of aging and is dramatically accelerated in diabetes where hyperglycemia is a major trigger (13, 15). Substantial evidence supports a role for AGE/RAGE interactions in the pathophysiology of diabetes (1, 2). Both AGEs and RAGE are upregulated in diabetic blood vessels, monocytes, and podocytes (16 - 19). Blockade of RAGE activation by recombinant sRAGE or function-blocking antibodies suppresses vascular hyperpermeability, atherosclerotic lesion development, and enhances wound healing in diabetic rodents (17, 19 - 21). Diabetic RAGE-null mice fail to develop signs of nephropathy including increased mesangial matrix expansion and thickening of the glomerular basement membrane (22). In contrast, transgenic over-expression of RAGE results in the exacerbation of diabetic nephropathy and retinopathy (23, 24).

RAGE is also a receptor for β-sheet fibrils, structures that are characteristic of amyloid. Aβ is a primary component of neurodegenerative plaques associated with Alzheimer's disease (AD), and elevated levels of RAGE are found co-localized with Aβ in AD brain (9, 25). Interaction of Aβ with RAGE expressed on endothelial cells, neurons, and microglia leads to the generation of reactive oxygen species and the production of proinflammatory factors, potential mechanisms underlying AD-related neurodegeneration (9). Recent evidence also suggests possible RAGE involvement in the transport of plasma Aβ across the blood-brain barrier and its subsequent accumulation in the CNS (26).

RAGE interactions with its ligand HMG-1 are shown to regulate cell motility. For instance, HMG-1/RAGE has the ability to stimulate neurite outgrowth in neuroblastoma cells, an activity that depends upon signaling by the GTPases Rac and Cdc42 (27). Blocking HMG-1/RAGE function has also been shown to suppress tumor growth and metastasis in animal models of cancer (28 - 30).

Several members of the S100 protein family are ligands for RAGE (5, 31). Binding of EN-RAGE/S100A12 or S100B induces signal transduction events mediated by NFκB, leading to induction of pro-inflammatory cytokines including TNF-α (5). Blockade of the EN-RAGE/RAGE pro-inflammatory axis suppresses delayed-type hypersensitivity reactions and inflammatory colitis in mouse models (5). In addition, both RAGE and S100 proteins are upregulated in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE), and the induction of EAE is suppressed when a dominant-negative form of RAGE is over-expressed (32).

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 the Quantikine Human RAGE kit can be used to determine relative mass values for naturally occurring RAGE.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for 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 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 standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by ligands, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

MATERIALS PROVIDED

Description	Part #	Cat. # DRG00	Cat. # SRG00
RAGE Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against RAGE.	892603	1 plate	6 plates
RAGE Conjugate - 21 mL/vial of polyclonal antibody against RAGE conjugated to horseradish peroxidase with preservatives.	892604	1 vial	6 vials
RAGE Standard - 50 ng/vial of recombinant human RAGE/Fc Chimera in a buffer with preservatives; lyophilized.	892605	1 vial	6 vials
Assay Diluent RD1-60 - 11 mL/vial of a buffered protein base with preservatives and blue dye.	895328	1 vial	6 vials
Calibrator Diluent RD5-5 - 21 mL/vial of a buffered protein base with preservatives. *For cell culture supernate samples.*	895485	1 vial	6 vials
Calibrator Diluent RD6-10 - 21 mL/vial of a buffered protein base with preservatives. *For serum/plasma samples.*	895468	1 vial	6 vials
Wash Buffer Concentrate - 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	895003	1 vial	6 vials
Color Reagent A - 12.5 mL/vial of stabilized hydrogen peroxide.	895000	1 vial	6 vials
Color Reagent B - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	6 vials
Stop Solution - 6 mL/vial of 2 N sulfuric acid.	895032	1 vial	6 vials
Plate Covers - Adhesive strips.	—	4 strips	24 strips

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). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="10">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="9">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Assay Diluent RD1-60</td></tr>
<tr><td>Calibrator Diluent RD5-5</td></tr>
<tr><td>Calibrator Diluent RD6-10</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*</td></tr>
</table>

*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.
- Test tubes for dilution.
- Human RAGE Controls (optional; available from R&D Systems).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

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.

SAMPLE COLLECTION AND STORAGE

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 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 heparin or EDTA 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

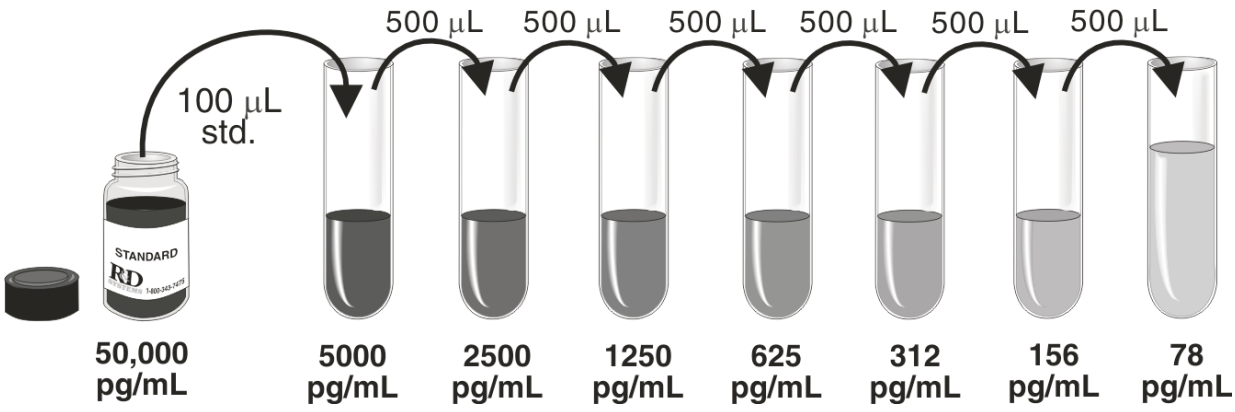
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into 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.

RAGE Standard - Reconstitute the RAGE Standard with 1.0 mL of 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 samples, controls, and standards be assayed in duplicate.

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 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. 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 200 µL of 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.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.

2. Add 100 µL Assay Diluent RD1-60 to each well.

3. Add 50 µL Standard, control or sample to each well. Incubate 2 hours RT.

4. Aspirate and wash 4 times.

5. Add 200 µL Conjugate to each well. Incubate 2 hours RT.

6. Aspirate and wash 4 times.

7. Add 200 µL Substrate Solution to each well. Incubate 30 minutes RT. **Protect from light.**

8. Add 50 µL Stop Solution to each well. Read at 450 nm within 30 minutes.
λ correction 540 or 570 nm

CALCULATION OF RESULTS

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

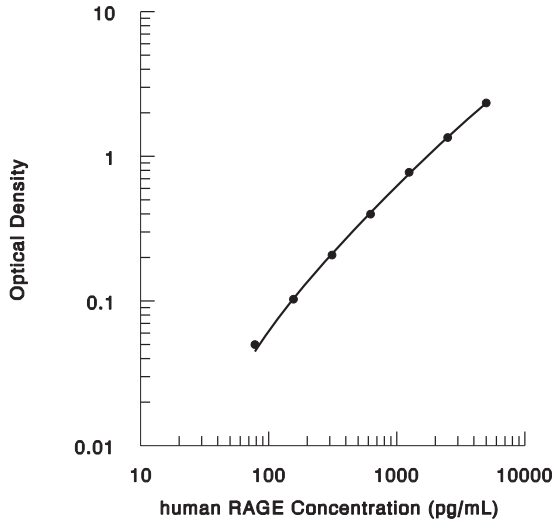
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best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

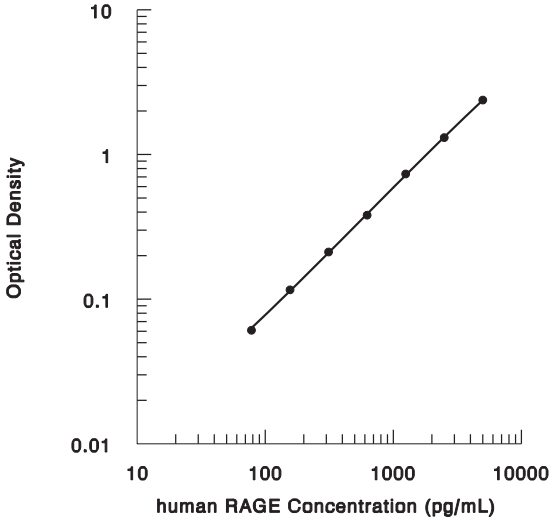
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Calibrator Diluent RD5-5



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
312	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

Calibrator Diluent RD6-10



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
312	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

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.
- 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.
- 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. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

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.

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 (%)	5.9	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	8.2	8.2	6.7

RECOVERY

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

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

LINEARITY

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

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

SENSITIVITY

Fifty-seven assays were evaluated and the minimum detectable dose (MDD) of 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 optical density value of twenty zero standard replicates and calculating the corresponding concentration.

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 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 calf 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 supernate were removed and assayed for levels of natural RAGE. No detectable levels of human RAGE were observed.

JE-3 cells were cultured in DMEM with 10% fetal calf serum and measured 648 pg/mL.

SPECIFICITY

This assay recognizes recombinant and natural 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:	**Natural protein:**
EN-RAGE	bovine AGE-BSA
HMG-1	
S100A10	
S100B	

Recombinant mouse and rat RAGE were found to have < 2% cross-reactivity in this assay.

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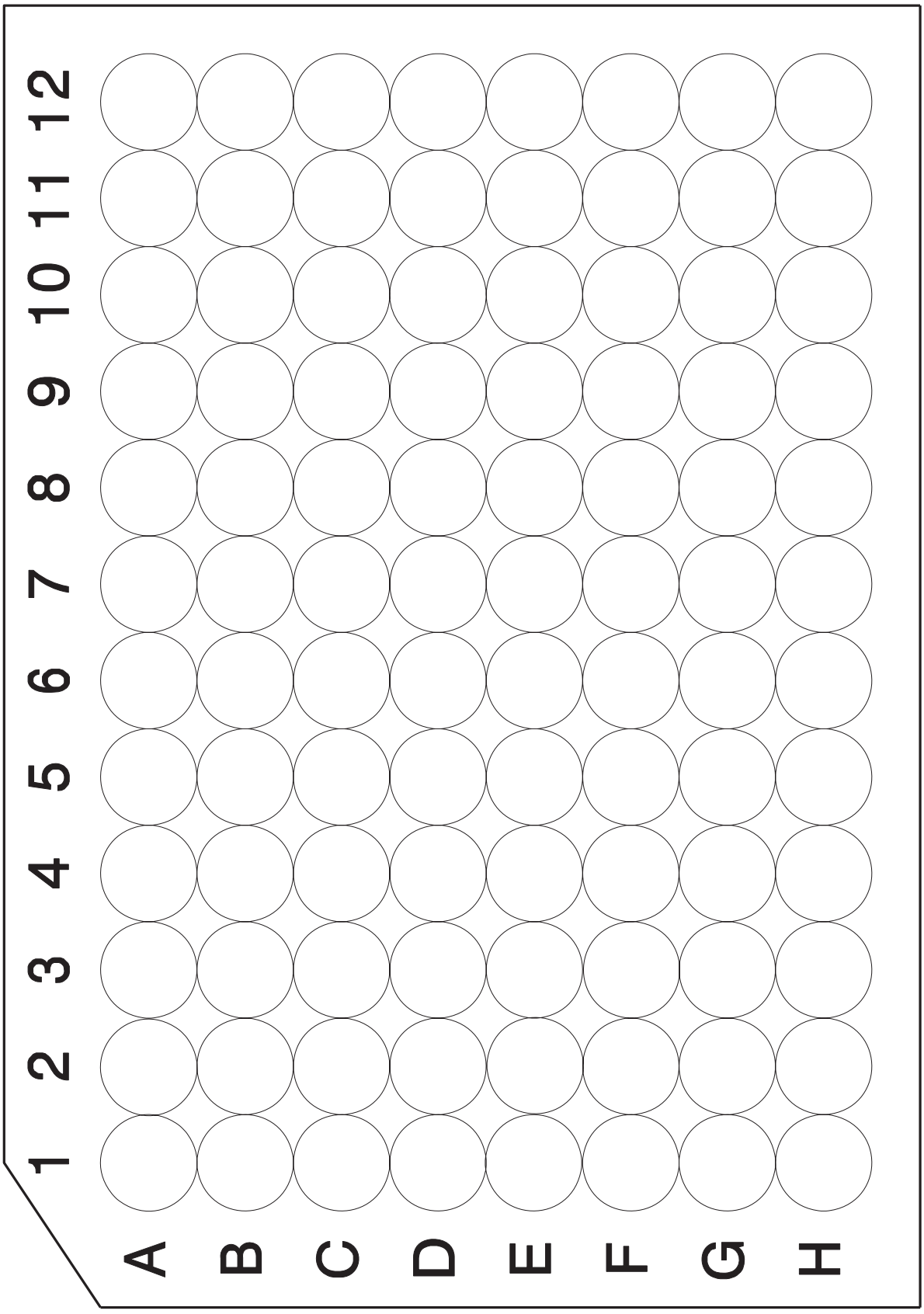
PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.



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