Quantikine®

Human EPCR Immunoassay

Catalog Number DEPCR0

For the quantitative determination of human Endothelial Protein C Receptor (EPCR) 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

Endothelial Protein C Receptor (EPCR), also known as activated protein C receptor, is a 49 kDa type I transmembrane glycoprotein that belongs to the CD1/major histocompatability complex class I family of molecules (1 - 3). It is synthesized as a 238 amino acid (aa) precursor with a 17 aa signal sequence, a 193 aa extracellular domain (ECD), a 21 aa transmembrane region, and a short 7 aa cytoplasmic region (SwissProt. # Q9UNN8). The ECD is made up of multiple α-helices and β-strands and contains the class I histocompatability antigen, domains α1 and α2 (amino acids 74 - 200), four potential N-linked glycosylation sites, and four cysteine residues. Human EPCR shares 73% and 69% aa sequence identity with bovine and mouse EPCR, respectively (2). The release of cell-surface EPCR, mediated by TACE/ADAM17, produces a 43 kDa soluble form of EPCR (4, 5). EPCR is expressed most prominently in large vessel endothelial cells (6, 7) but is also detected in neutrophils (8) and vascular smooth muscle cells (9).

Both the cell surface and soluble forms of EPCR bind the plasma vitamin K-dependent zymogen, protein C, and the anticoagulant serine protease, activated protein C (APC), with high affinity (1, 3) and have activities that impact coagulation, inflammation, fibrinolysis, and cell proliferation (10). The protein C pathway is clinically important, as evidenced by the multitude of dysfunctions in this pathway that result in thrombosis (11, 12). Activation of protein C to APC on the surface of endothelial cells initiates a series of events that play key roles in the regulation of blood coagulation and in the host response to inflammation (4). Protein C is transformed to its active form by thrombin-mediated cleavage at the N-terminus (10). Effective activation of protein C by thrombin requires the transmembrane glycoprotein, thrombomodulin (TM), as a cofactor for thrombin, amplifying this event greater than 1000-fold (10). When thrombin is complexed with TM, it has reduced pro-coagulant activity as shown by its decreased ability to cleave fibrinogen, activate factor V, and trigger platelet activation (10). When protein C is bound to EPCR, protein C activation by the thrombin-TM complex is further enhanced 20-fold and is concentrated near the surface of the vessel wall (10, 13). When APC is generated, it remains bound to EPCR for a short time before associating with protein S on the surface of platelets or endothelium, whereon it is inactivated by α1-antitrypsin, the protein C inhibitor, and/or α2-macroglobulin (10). In addition to its role of amplifying activation of protein C, EPCR switches the substrate specificity of APC (10). When APC is released from EPCR, it has anticoagulant properties, yet when transiently complexed with EPCR, APC cleaves protease activated receptor 1 (PAR1), initiating intracellular signaling that provides anti-apoptotic protection (10, 14, 15) and sets off its anti-inflammatory activities (10). Unlike membrane bound EPCR, which enhances protein C activation, soluble EPCR blocks cellular protein C activation and APC anticoagulant activity (16). It thus has an opposite effect on the protein C coagulation pathway. EPCR is highly regulated. Thrombin increases metalloprotease activity and also causes a rapid increase in EPCR mRNA (17, 18). Circulating levels of EPCR are increased, and the cellular EPCR is replenished by the upregulation of EPCR mRNA.

The Quantikine Human EPCR Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human EPCR in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant human EPCR and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human EPCR showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Human EPCR kit can be used to determine relative mass values for naturally occurring EPCR.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for EPCR has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any EPCR present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for EPCR 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 EPCR 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.
- 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

EPCR Microplate (Part 893276) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against EPCR.

EPCR Conjugate (Part 893277) - 12 mL of a polyclonal antibody against EPCR conjugated to horseradish peroxidase with preservatives.

EPCR Standard (Part 893278) - 80 ng of recombinant human EPCR in a buffered protein base with preservatives; lyophilized.

Assay Diluent RD1-17 (Part 895433) - 11 mL of a buffered protein base with preservatives.

Calibrator Diluent RD5-24 Concentrate (Part 895325) - 21 mL of a buffered protein base with preservatives.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895174) - 23 mL of diluted hydrochloric acid.

Plate Covers - 4 Adhesive strips.

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="9">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="8">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Assay Diluent RD1-17</td></tr>
<tr><td>Calibrator Diluent RD5-24</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

- Hydrochloric acid (A.C.S. Grade, 12 N)
- Sodium hydroxide (A.C.S. Grade, 10 N)
- 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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- 100 mL and 500 mL graduated cylinders.
- Human EPCR Controls (optional; available from R&D Systems).

PRECAUTION

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

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 10 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, EDTA, or citrate as an anticoagulant. Centrifuge for 10 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.

PRETREATMENT REAGENT PREPARATION

Note: *Do not pretreat the kit standard.*

Prepare the following solutions for acid treatment and neutralization. The solutions may be stored in polypropylene bottles at room temperature for up to one month.

Caution: *Wear protective clothing and safety glasses while preparing or using these reagents. Always add acid to water.*

1N HCl (100 mL) - To 91.67 mL of deionized water, slowly add 8.33 mL of 12N HCl. Mix well.

1N NaOH (100 mL) - To 90 mL of deionized water, slowly add 10 mL of 10N NaOH. Mix well.

For each new lot of reagents, measure the pH of several representative samples after neutralization to ensure that it is within pH 5.0 - 6.0. Adjust the volume and corresponding dilution factor of the 1N NaOH as needed.

SAMPLE PRETREATMENT PROCEDURE

Assay samples immediately after neutralization (pH 5.0 - 6.0). Do not store acid treated samples.

Cell Culture Supernate Samples	Serum/Plasma Samples
To 100 µL of cell culture supernate, add 50 µL of 1N HCl.	To 40 µL serum/plasma, add 20 µL of 1N HCl
Mix well.	Mix well.
Incubate 10 minutes at room temperature.	Incubate 10 minutes at room temperature.
Neutralize the acidified sample by adding 45 µL of 1N NaOH.	Neutralize the acidified sample by adding 18 µL of 1N NaOH.
Mix well.	Mix well.
Assay immediately.	Prior to the assay, dilute the acid treated sample 20-fold with Calibrator Diluent RD5-24 (1X) by adding 20 µL of acid treated sample to 380 µL of Calibrator Diluent RD5-24 (1X).
The concentration read off the standard curve must be multiplied by the dilution factor, 1.95.	The concentration read off the standard curve must be multiplied by the dilution factor, 39.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

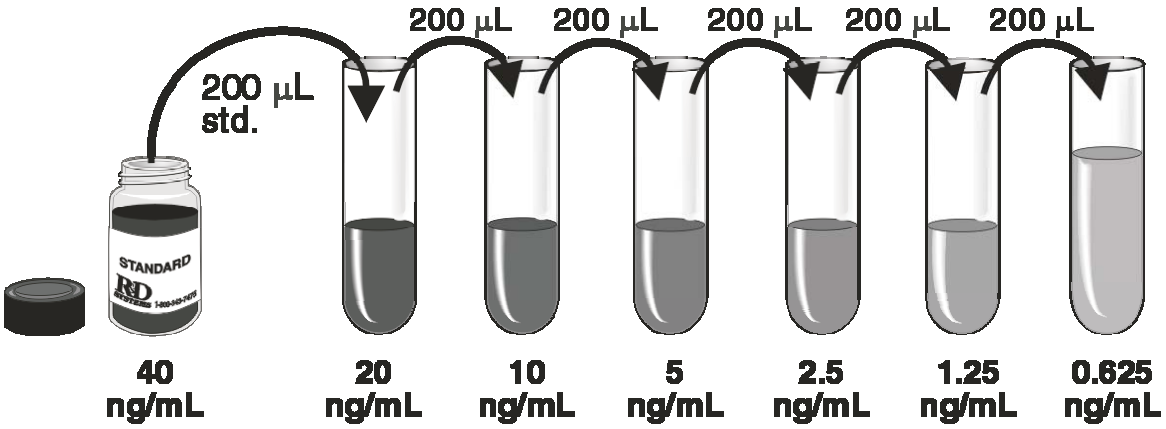
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD5-24 (1X) - Dilute 10 mL of Calibrator Diluent RD5-24 Concentrate into 40 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5-24 (1X).

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.

EPCR Standard - Reconstitute the EPCR Standard with 2.0 mL of Calibrator Diluent RD5-24 (1X). This reconstitution produces a stock solution of 40 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5-24 (1X) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 40 ng/mL standard serves as the high standard. Calibrator Diluent RD5-24 (1X) 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, standards, and controls 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, reseal.
3. Add 50 µL of Assay Diluent RD1-17 to each well.
4. Add 50 µL of Standard, sample*, or control per well. Pipette samples within 15 minutes. 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 toweling.
6. Add 100 µL of EPCR 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. If 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 require pretreatment. See Sample Pretreatment Procedure.

ASSAY PROCEDURE SUMMARY

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

2. Add 50 µL of Assay Diluent RD1-17 to each well.

3. Add 50 µL of Standard, sample*, or control to each well. Incubate for 2 hours at RT on the shaker.

4. Aspirate and wash 4 times.

5. Add 100 µL of Conjugate to each well. Incubate for 2 hours at RT on the shaker.

6. Aspirate and wash 4 times.

7. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at RT **on the benchtop. Protect from light.**

8. Add 100 µL of Stop Solution to each well. Read at 450 nm within 30 minutes. λ correction 540 or 570 nm

*Samples require pretreatment. See Sample Pretreatment Procedure.

CALCULATION OF RESULTS

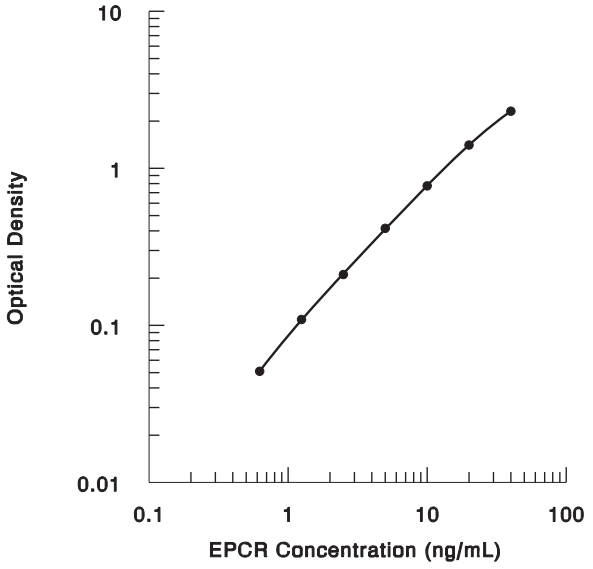
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 EPCR concentrations versus the log of the O.D. and the best fit line can be determined by regession analysis. This procedure will produce an adequate but less precise fit of the data.

Since 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	0.029 0.029	0.029	—
0.625	0.080 0.080	0.080	0.051
1.25	0.137 0.138	0.138	0.109
2.5	0.236 0.243	0.240	0.211
5	0.444 0.445	0.444	0.415
10	0.801 0.802	0.802	0.773
20	1.433 1.439	1.436	1.407
40	2.337 2.348	2.342	2.313

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.
- It is recommended that samples be pipetted within 15 minutes.
- 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 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. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.
- 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.

PRECISION

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

Inter-assay Precision (Precision between assays)
Three samples of known concentration were assayed in forty separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	5.20	11.7	21.5	5.26	10.7	21.0
Standard deviation	0.2	0.6	1.7	0.3	0.7	1.3
CV (%)	4.8	4.9	7.7	5.4	6.4	6.0

RECOVERY

The recovery of EPCR spiked to three levels in samples throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture samples* (n=6)	93	86 - 99%

*Samples were acid treated prior to assay.

LINEARITY

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

		Cell culture samples* (n=6)	Serum* (n=6)	Heparin plasma* (n=6)	EDTA plasma* (n=6)	Citrate plasma* (n=6)
1:2	Average % of Expected	94	103	101	105	103
	Range (%)	90 - 99	99 - 107	96 - 107	102 - 108	98 - 107
1:4	Average % of Expected	99	105	103	106	101
	Range (%)	92 - 104	100 - 107	99 - 110	102 - 110	97 - 105
1:8	Average % of Expected	100	104	104	107	99
	Range (%)	94 - 104	99 - 108	100 - 110	104 - 109	93 - 103
1:16	Average % of Expected	95	100	99	100	94
	Range (%)	88 - 103	93 - 104	92 - 104	96 - 107	86 - 101

*Samples were pretreated and then diluted prior to assay.

SENSITIVITY

Seventy-five assays were evaluated and the minimum detectable dose (MDD) of EPCR ranged from 0.014 - 0.282 ng/mL. The mean MDD was 0.064 ng/mL.

The minimum detectable dose 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 EPCR produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples drawn from apparently healthy volunteers were evaluated for the presence of EPCR 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=36)	528	270 - 1435	282
Heparin plasma* (n=36)	519	249 - 1596	300
EDTA plasma* (n=36)	510	243 - 1535	286
Citrate plasma* (n=11)	579	289 - 1073	298

*Samples were pretreated and then diluted prior to assay.

Cell Culture Supernates -
Human peripheral blood leukocytes (PBLs; 1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 50 μM β-mercaptoethanol, 10 ng/mL rhIL-2, 10 μg/mL PHA, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate for 5 days. An aliquot of the cell culture supernate was removed, pretreated, assayed for levels of EPCR, and measured 3.27 ng/mL.

Human umbilical vein endothelial cells (HUVEC; 2 x 10^4 cells/mL) were cultured in EGM®-2 media for 3 days. An aliquot of the cell culture supernate was removed, pretreated, assayed for levels of EPCR, and measured 22.2 ng/mL.

Human colon adenocarcinoma (HT-29) cells were cultured in McCoy's 5a media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate for 7 days. An aliquot of the cell culture supernate was removed, pretreated, assayed for levels of human EPCR, and measured 85.0 ng/mL.

EGM is a registered trademark of Lonza.

SPECIFICITY

This assay recognizes recombinant and natural human EPCR. The factors listed below were prepared Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors prepared in a mid-range recombinant human EPCR control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:	**Recombinant mouse:**	**Natural protein:**
Factor X	Protein C	human Prothrombin (Factor II)
Factor Xa	Thrombomodulin	
Protein C		
Thrombin		
Thrombomodulin		

Recombinant human Protein S shows 0.004% cross-reactivity in this assay.

Recombinant mouse EPCR shows < 2% cross-reactivity in this assay.

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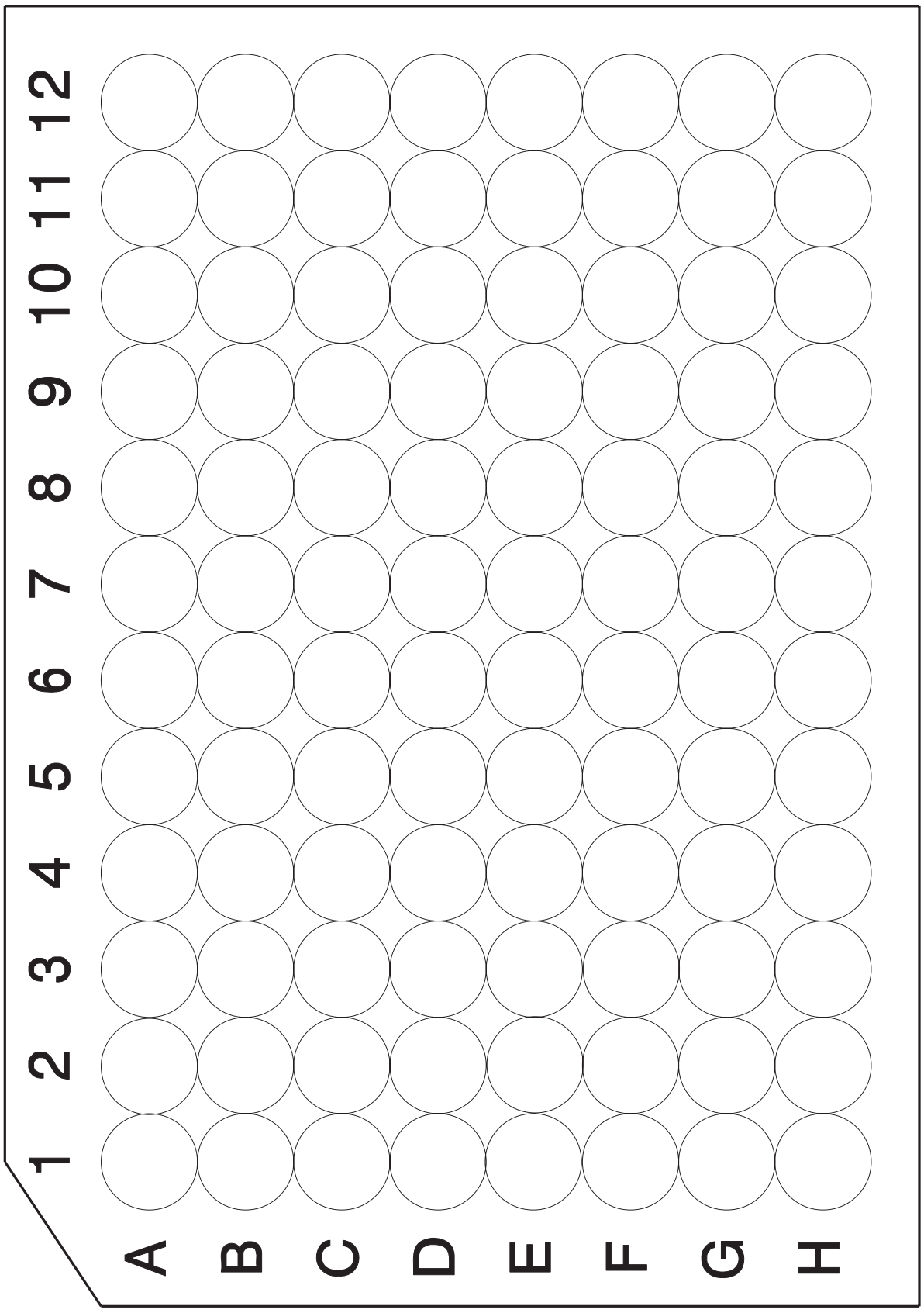
PLATE LAYOUT

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



NOTES