Quantikine®

Porcine IL-6 Immunoassay

Catalog Number P6000

For the quantitative determination of porcine interleukin 6 (IL-6) 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

Interleukin 6 (IL-6) is a 26 kDa multifunctional cytokine that plays an important role in host defense, acute phase reactions, immune responses, nerve cell function, hematopoiesis and bone remodeling (1 - 6). IL-6 is produced by a number of normal and transformed lymphoid and nonlymphoid cell types. Its production is upregulated by numerous signals such as mitogenic or antigenic stimulation, lipopolysaccharide, calcium ionophores, cytokines and viruses. Its expression is inhibited in monocytes by cytokines such as IL-4, IL-10 and IL-13. Elevated serum IL-6 levels have been observed in a number of pathological conditions, including bacterial and viral infections, trauma, autoimmune disease, inflammation, and malignancy (1 - 6).

IL-6 is a prototypic member of the IL-6 superfamily of cytokines that utilize gp130 as their signal transducing receptor subunit (4). The cDNAs for porcine, human, and mouse IL-6 have all been cloned (7, 8). Porcine IL-6 cDNA encodes a 212 amino acid (aa) precursor polypeptide that contains a 29 aa signal sequence with a 183 aa mature segment (7, 8). Mature porcine IL-6 shares 58% and 39% aa identity with human and mouse IL-6, respectively (7). Porcine IL-6 is reported to be active on both human and mouse cells (7, 9). To date, the number of porcine cell types reported to express IL-6 are limited and include fibroblasts (7), macrophages (10), and endothelial cells (11).

The high-affinity IL-6 receptor complex that mediates IL-6 bioactivity consists of two membrane glycoproteins: an 80 kDa low-affinity IL-6 binding receptor termed IL-6 Rα, and a 130 kDa signal transducing subunit termed gp130 that lacks IL-6 binding ability (4). On the cell surface, IL-6 first binds IL-6 Rα and then recruits gp130 into a trimeric complex. Two trimeric complexes subsequently associate to form the high affinity hexameric signaling unit (12 - 14).

The Quantikine Porcine IL-6 Immunoassay is a 4.5 hour solid phase ELISA designed to measure porcine IL-6 in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant porcine IL-6 and antibodies raised against the recombinant factor. The immunoassay has been shown to accurately quantitate recombinant porcine IL-6. Results obtained using natural porcine IL-6 show dose response curves that run parallel to standard curves obtained using recombinant Quantikine Porcine IL-6 kit standards. These results indicate that the Quantikine Porcine IL-6 Immunoassay kit can be used to determine relative mass values for natural porcine IL-6.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for porcine IL-6 has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any porcine IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for porcine IL-6 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 porcine IL-6 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, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in 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 soluble receptors, binding proteins, and other factors present in biological samples. Until all receptors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

PRECAUTIONS

Calibrator Diluent RD6-32 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. Wear eye, hand, face, and clothing protection when using this material.

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.
- For best results, pipette reagents and samples into the center of each well.
- It is recommended that the samples be pipetted within 15 minutes.
- 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.

MATERIALS PROVIDED

Porcine IL-6 Microplate (Part 890865) - 96 well polystyrene microplate (12 strips of 8 wells) coated with polyclonal antibody specific for porcine IL-6.

Porcine IL-6 Conjugate (Part 890867) - 23 mL of a polyclonal antibody against porcine IL-6 conjugated to horseradish peroxidase with preservatives.

Porcine IL-6 Standard (Part 890866) - 3 vials (5 ng/vial) of recombinant porcine IL-6 in a buffered protein base with preservatives; lyophilized.

Porcine IL-6 Control (Part 890187) - 3 vials of recombinant porcine IL-6 in a buffered protein base with preservatives; lyophilized. The concentration range of porcine IL-6 after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.

Assay Diluent RD1-63 (Part 895352) - 12.5 mL of a buffered protein solution with preservatives.

Calibrator Diluent RD5T (Part 895175) - 21 mL of a buffered protein solution with preservatives. *For cell culture supernate samples.*

Calibrator Diluent RD6-32 (Part 895336) - 21 mL of diluted animal serum with preservatives. *For serum/plasma samples.*

Wash Buffer Concentrate (Part 895024) - 50 mL of a 25-fold concentrated solution of a 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 a diluted hydrochloric acid solution.

Plate Covers (Part 640197) - 4 adhesive plate sealers.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use beyond kit expiration date.</td></tr>
<tr><td rowspan="10"></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>Calibrator Diluent RD5T</td></tr>
<tr><td>Calibrator Diluent RD6-32</td></tr>
<tr><td>Assay Diluent RD1-63</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>Porcine IL-6 Standard (2500 pg/mL)</td><td rowspan="2">Use a new Standard and Control for each assay.</td></tr>
<tr><td>Porcine IL-6 Control</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.
- 100 mL and 1000 mL graduated cylinders.
- **Polypropylene test tubes.**

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 - Allow blood samples to clot for 2 hours at room temperature or overnight at 2 - 8° C before centrifuging for 20 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 as an anticoagulant. Centrifuge 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: *Grossly hemolyzed or lipemic samples may not be suitable for measurement of porcine IL-6 with this assay.*

SAMPLE PREPARATION

Serum and plasma samples require a 2-fold dilution into Calibrator Diluent RD6-32 prior to assay. A suggested 2-fold dilution is 125 µL sample + 125 µL Calibrator Diluent RD6-32.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

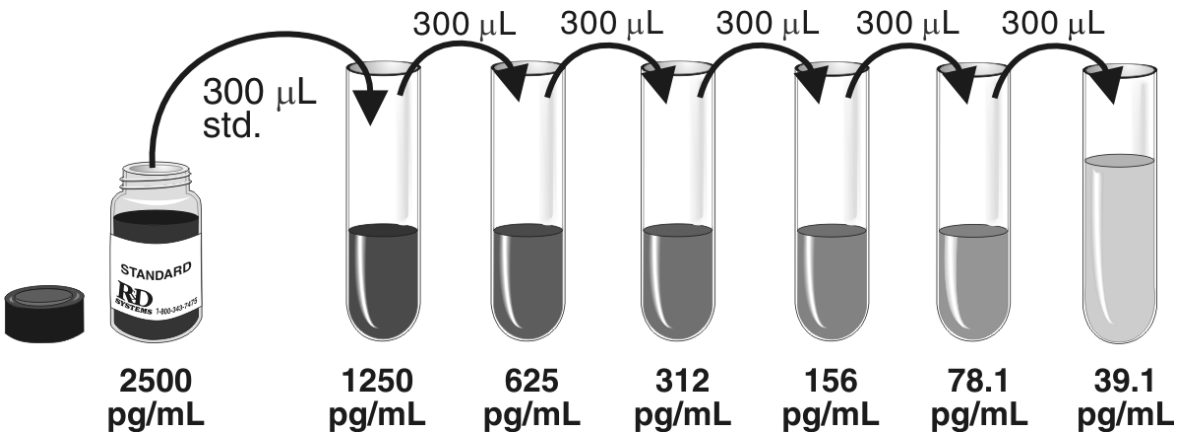
Porcine IL-6 Kit Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL Wash Buffer Concentrate into deionized or distilled water to prepare 625 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. 120 µL of the resultant mixture is required per well.

Porcine IL-6 Standard - Reconstitute the Porcine IL-6 Standard with 2.0 mL of Calibrator Diluent RD5T *(for cell culture supernate samples)* or Calibrator Diluent RD6-32 *(for serum/plasma samples)*. Do not substitute other diluents. This reconstitution produces a stock solution of 2500 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions. Mix each tube thoroughly before the next transfer.

Use polypropylene tubes. Pipette 300 µL of the appropriate Calibrator Diluent into each tube. Use the stock solution to produce a dilution series (below). The undiluted porcine IL-6 Standard serves as the high standard (2500 pg/mL). 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, standards, and control be assayed in duplicate.

1. Prepare reagents, samples, standards, and control 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 RD1-63 to each well.

4. Add 100 µL of Standard, Control, or sample* per well. Mix by gently tapping the plate frame for 1 minute. 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 four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, pipette, 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 Porcine IL-6 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 120 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

9. Add 120 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.

*Porcine serum and plasma samples require a 2-fold dilution. See Sample Preparation.

PROCEDURE SUMMARY AND CHECKLIST

1. ☐ Bring all reagents to room temperature.
☐ Prepare reagents, standards, and samples as instructed.
☐ Return unused components to storage temperature as indicated in the instructions.

2. ☐ Add 50 µL Assay Diluent to each well.

3. ☐ Add 100 µL Standard, Control, or sample* to each well.
☐ Tap plate gently for one minute.
☐ Cover the plate and incubate for 2 hours at room temperature.

4. ☐ Aspirate and wash each well five times.

5. ☐ Add 200 µL Conjugate to each well.
☐ Cover the plate and incubate for 2 hours at room temperature.

6. ☐ Aspirate and wash each well five times.

7. ☐ Add 120 µL Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

8. ☐ Add 120 µL Stop Solution to each well.

9. ☐ Read Optical Density at 450 nm (correction wavelength set at 540 nm or 570 nm).

*Porcine serum/plasma samples require dilution.

CALCULATION OF RESULTS

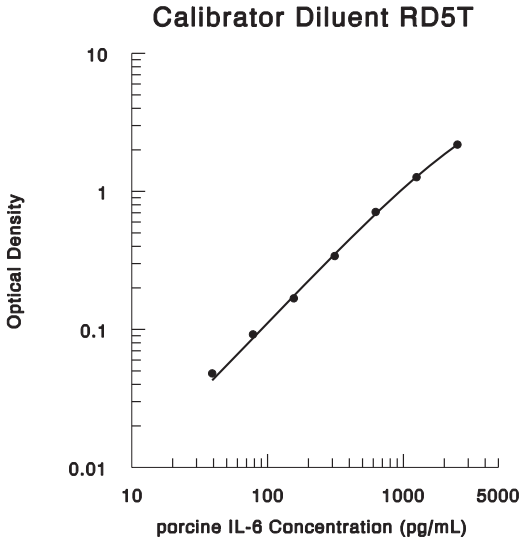
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 porcine IL-6 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.

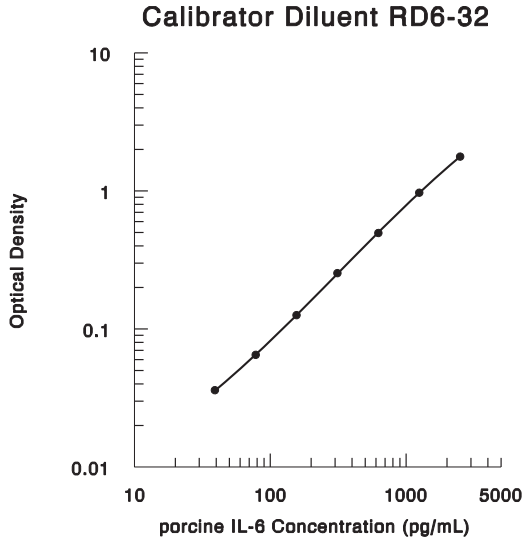
Because serum and plasma samples have been diluted prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

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



(pg/mL)	O.D.	Average	Corrected
0	0.066 0.067	0.066	—
39.1	0.115 0.113	0.114	0.048
78.1	0.160 0.155	0.158	0.092
156	0.233 0.235	0.234	0.168
312	0.398 0.413	0.406	0.340
625	0.787 0.766	0.776	0.710
1250	1.336 1.330	1.333	1.267
2500	2.181 2.320	2.250	2.184



(pg/mL)	O.D.	Average	Corrected
0	0.065 0.068	0.066	—
39.1	0.102 0.101	0.102	0.036
78.1	0.128 0.134	0.131	0.065
156	0.194 0.191	0.192	0.126
312	0.319 0.322	0.320	0.254
625	0.563 0.561	0.562	0.496
1250	1.030 1.044	1.037	0.971
2500	1.819 1.859	1.839	1.773

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 assays to assess inter-assay precision.

Serum/Plasma Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	151	385	1445	152	391	1429
Standard deviation	4.4	13.6	31.7	15.2	31.7	105
CV (%)	2.9	3.5	2.2	10.0	8.1	7.3

Cell Culture Supernate Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	141	333	1196	140	341	1209
Standard deviation	9.2	12.5	22.1	9.4	19.3	53.7
CV (%)	6.5	3.8	1.8	6.7	5.6	4.4

RECOVERY

The recovery of porcine IL-6 spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n = 9)	97	80 - 104%
Porcine serum* (n = 7)	95	80 - 107%
Porcine heparin plasma* (n = 4)	101	94 - 107%

*Porcine serum/plasma samples were diluted 2-fold prior to assay as directed in the Sample Preparation section.

LINEARITY

To assess the linearity of the assay, five or more samples containing and/or spiked with high concentrations of porcine IL-6 in each matrix were diluted with the appropriate Calibrator Diluent and then assayed. Results from typical sample dilutions are shown.

Sample	Dilution	Observed (pg/mL)	Expected (pg/mL)	$\frac{\text{Observed}}{\text{Expected}} \times 100$
Cell Culture Supernates	spiked	1038		
	1:2	518	519	100
	1:4	250	260	96
	1:8	123	130	95
	1:16	64	65	98
Porcine Serum*	spiked	1111		
	1:2	546	556	98
	1:4	265	278	95
	1:8	132	139	95
	1:16	69	69	100
Porcine Heparin Plasma*	spiked	1094		
	1:2	554	547	101
	1:4	276	274	101
	1:8	150	137	109
	1:16	74	68	109

*Porcine serum/plasma samples were diluted 2-fold, as directed in the Sample Preparation section.

SENSITIVITY

The minimum detectable dose of porcine IL-6 is typically 10 pg/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 *E. coli*-expressed recombinant porcine IL-6 produced at R&D Systems. This recombinant form of porcine IL-6 contains 184 amino acid residues and has a predicted molecular mass of 21 kDa.

Based on total amino acid analysis, the absorbance of a 1 mg/mL solution of the *E. coli*-expressed recombinant porcine IL-6 at 280 nm was determined to be 0.72 A.U.

SAMPLE VALUES

Serum - Twenty-four individual porcine serum samples were evaluated for detectable levels of porcine IL-6 in this assay. All samples measured below the lowest standard, 39.1 pg/mL.

Plasma - Twelve individual porcine heparin plasma samples were evaluated for detectable levels of porcine IL-6 in this assay. Eleven samples measured about or below the lowest standard, 39.1 pg/mL. One sample read 2352 pg/mL.

Cell Culture Supernates - Porcine PBL cells (5 x 10^6 cells/mL) were cultured for 6 days in DMEM supplemented with 10% fetal bovine serum and stimulated twice with 100 ng/mL lipopolysaccharide at day 0 and day 4. The cell culture supernate was assayed for porcine IL-6 and measured 4 ng/mL.

SPECIFICITY

This assay recognizes both recombinant and natural porcine IL-6. The factors listed below were prepared at 50 ng/mL in Calibrator Diluents RD5T and RD6-32 and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range porcine IL-6 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant porcine:	**Recombinant human:**	**Recombinant mouse:**	**Recombinant rat:**	**Other:**
IL-1α	IL-6	IL-6	IL-6	porcine PDGF
IL-1β	IL-6 sR			
IL-2	IL-6 Rα/gp130			
IL-4				
IL-8				
IL-10				
TGF-β				
TNF-α				

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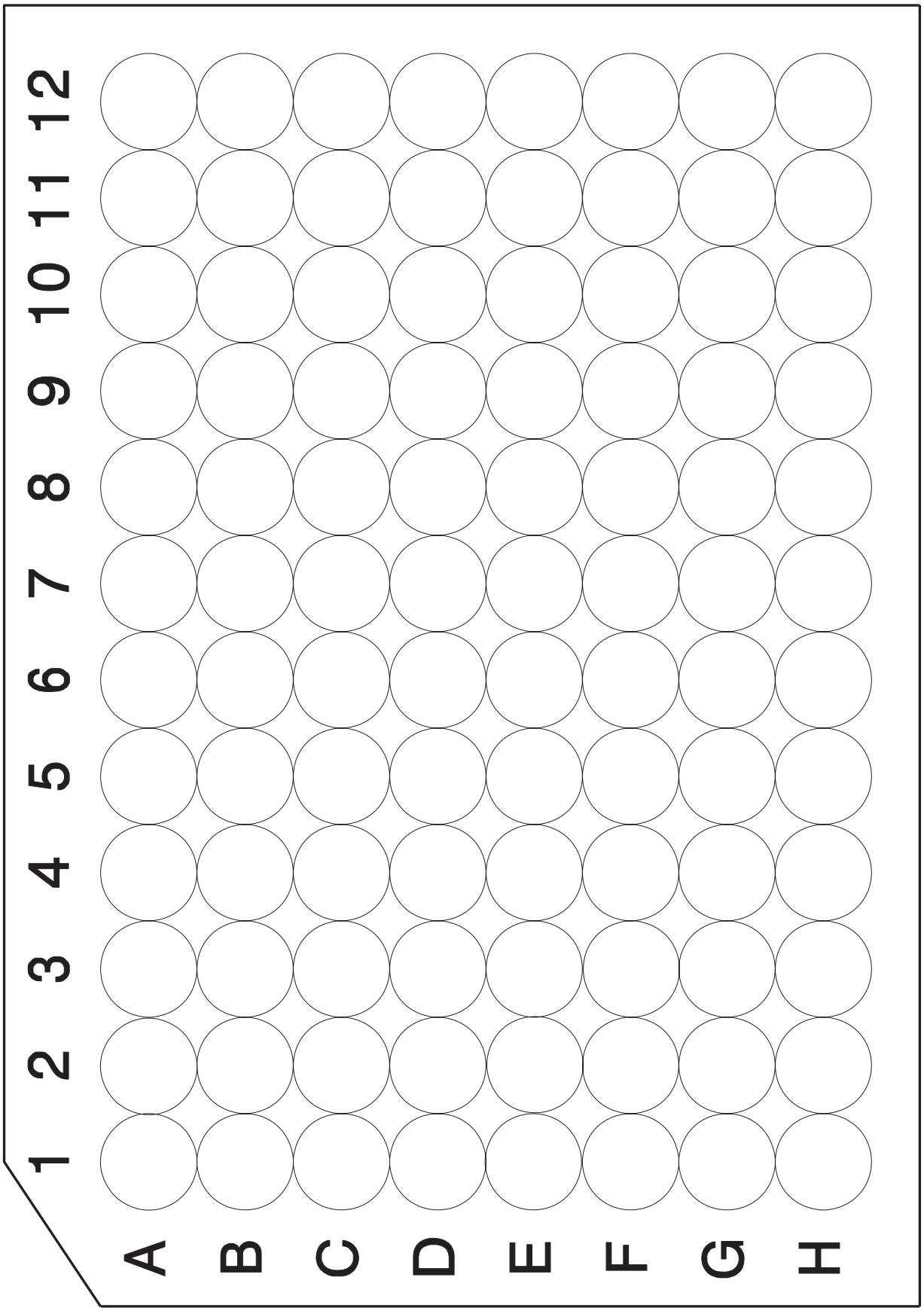
PLATE LAYOUT

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



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