Quantikine™ QuicKit™ ELISA

Human PD-1 Immunoassay

Catalog Number QK1086

For the quantitative determination of human Programmed Death-1 (PD-1) concentrations in cell culture supernates, cell lysates, 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

Programmed Death-1 (PD-1), also known as Programmed cell death protein and CD279, is an extensively studied immune checkpoint inhibitory receptor. Given PD-1's role in peripheral tolerance, it is not surprising that increased PD-1 expression is a mechanism for immune escape, which is permissive for cancer growth and metastasis (1,2). PD-1 is encoded by the *PDCD1* gene (3). The PD-1 glycoprotein is a monomeric 50-55kDa type 1 transmembrane protein that belongs to the immunoglobulin (Ig) superfamily (4). PD-1 expression and induction have been well studied (3,5,6). It is expressed in $CD4^+$ and $CD8^+$ T cells as well as B cells, macrophages, some dendritic cell subsets and NK cells. PD-1 is induced by T cell receptor (TCR) signaling as well as interleukin 2 (IL-2), IL-7 and type 1 interferons. A variety of reagents are used to experimentally induce PD-1 expression, including phorbol 12-myristate 13 acetate, ionomycin, concanavalin A, CD3/CD28 antibodies, and most notably lymphocytic choriomeningitis virus *in vivo* (6).

PD-1 expression is also regulated post-translationally (2). The PD-1 extracellular domain is glycosylated at asparagine (N) residues N49 and N72 while in the endoplasmic reticulum. PD-1 subsequently transits to the golgi apparatus where it is fucosylated at the same sites by the core fucosylase FUT8. This post-translational modification represents a novel therapeutic target as T cells have a more robust anticancer response due to reduced surface expression of de-fucosylated PD-1. PD-1 is polyubiquinated at lysine (K) residue K48 by the E3 ligase F-Box 38 (FBX028) (2,7) which results in PD-1 degradation via the proteosome.

PD-1 serves as the receptor for programmed death ligand 1 (PD-L1/CD274/B7-H1) and 2 (PD-L2 CD273/B7-DC) (8,9). The interaction of the extracellular domains of PD-1 and PD-L1 causes a confirmational change that results in the phosphorylation of the cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM) by Src family kinases (4). Src homology 2 containing tyrosine phosphatase 2 (SHP-2) and SHP-1 are recruited in order to attenuate T cell activating signals via dephosphorylation of downstream signaling cascades.

The Quantikine™ QuicKit™ Programmed Death-1 (PD-1) Immunoassay is a one step, 80-minute solid phase ELISA designed to measure human PD-1 levels in cell culture supernates, cell lysates, serum, and plasma. It contains HEK293-expressed recombinant human PD-1 and antibodies raised against the recombinant protein. Results obtained using natural human PD-1 showed linear curves that were parallel to the standard curves obtained using the QuicKit standards. These results indicate that this kit can be used to determine relative mass values for natural human PD-1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An anti-tag antibody has been pre-coated onto a microplate. Standards and samples are pipetted into the wells followed by an antibody cocktail. The antibody cocktail consists of an affinity tag labeled monoclonal capture antibody and an enzyme-linked monoclonal detection antibody, specific for human PD-1. After washing away any unbound substances, a substrate solution is added to the wells and color develops in proportion to the amount of PD-1 bound. 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 from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with the 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™ QuicKit™ 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.
- Ensure reagent addition to plate wells is uninterrupted.
- To ensure accurate results, proper adhesion of the plate sealer during the incubation step 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
QuicKit™ Coated Microplate	899063	96 well polystyrene microplate (12 strips of 8 wells) coated with an anti-tag monoclonal antibody.	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 PD-1 Standard	899332	2 vials of recombinant human PD-1 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard for each assay. Discard after use.
Human PD-1 Capture Ab Concentrate	899330	Lyophilized tagged monoclonal antibody specific for human PD-1.	May be stored for up to 1 month at 2-8 °C.*
Human PD-1 Detection Ab Concentrate	899331	400 µL of a monoclonal antibody specific for human PD-1 conjugated to horseradish peroxidase with preservatives.	
Assay Diluent RD1-54	895321	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-17	895512	21 mL of a buffered protein base with preservatives.	
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	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

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 SDS on our website prior to use.

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
- 50 mL and 500 mL graduated cylinders
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards
- Human PD-1 Controls (optional; R&D Systems®, Catalog # QC296)

For cell lysates samples (optional):

- Lysis Buffer 17 (R&D Systems, Catalog # 895943)

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.

Cell Lysates - Cells must be lysed prior to assay as directed in the Sample Values section.

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.*

SAMPLE PREPARATION

Serum, plasma, and cell culture supernates can be tested neat. Some supernates may require dilution due to high endogenous levels.

Multiple dilutions are recommended for unknown samples.

For cell lysate samples, quantitation of sample protein concentration using a total protein assay is recommended. The suggested range for total cell lysate protein added is 1-35 µg/well.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human PD-1 Capture Ab Concentrate - **Refer to the vial label for reconstitution volume.** Reconstitute the Human PD-1 Capture Ab Concentrate with Assay Diluent RD1-54. This reconstitution produces a 20X Capture Antibody stock. Allow the capture antibody to sit for a minimum of 5 minutes with gentle agitation prior to diluting. Once reconstituted, the 20X Capture Ab stock can be stored for 4 weeks at 2-8 °C.

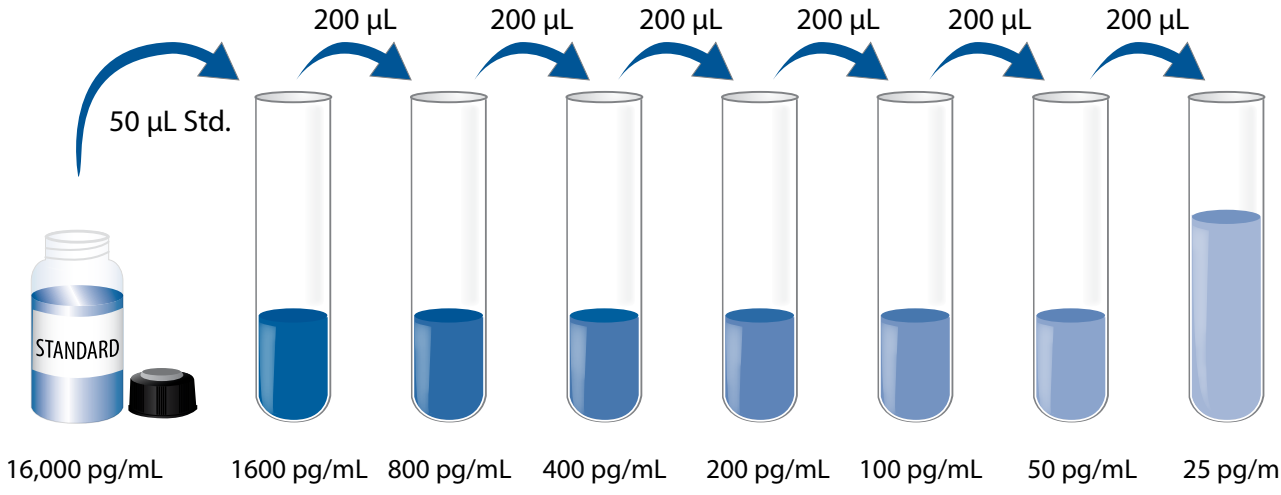
Antibody Cocktail - Dilute the reconstituted Capture Ab stock and the Detection Ab Concentrate 20-fold in Assay Diluent RD1-54. For a full plate, add 300 µL of reconstituted Human PD-1 Capture Ab Concentrate and 300 µL of Human PD-1 Detection Ab Concentrate to 5.4 mL of Assay Diluent RD1-54.

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. 100 µL of the resultant mixture is required per well.

Human PD-1 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human PD-1 Standard with deionized or distilled water. This reconstitution produces a stock solution of 16,000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5-17 into the 1600 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 1600 pg/mL standard serves as the high standard. Calibrator Diluent RD5-17 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, 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 50 µL of standard, control, or sample* per well. A plate layout is provided to record standards and samples assayed.
4. Add 50 µL of Antibody Cocktail to each well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process twice for a total of three 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 Substrate Solution to each well. Incubate for 20 minutes at room temperature **on the benchtop. Protect from light.**
7. 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.
8. 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

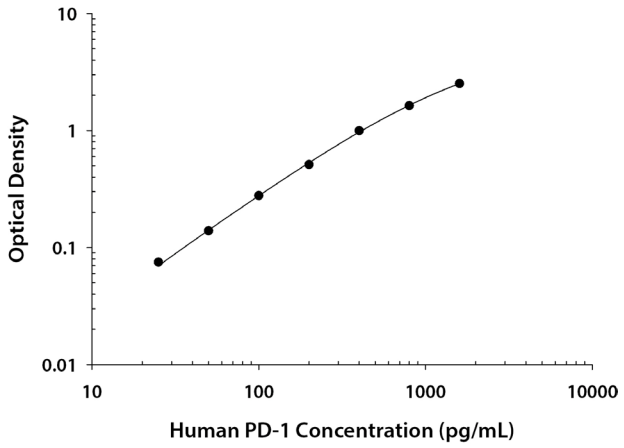
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human PD-1 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	0.028 0.028	0.028	——
25	0.099 0.106	0.103	0.075
50	0.163 0.170	0.167	0.139
100	0.304 0.307	0.306	0.278
200	0.536 0.543	0.540	0.512
400	1.010 1.037	1.024	0.996
800	1.630 1.688	1.659	1.631
1600	2.528 2.567	2.548	2.520

PRECISION

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

Inter-Assay Precision (Precision between assays)
Two samples of known concentration were tested in ten separate assays to assess inter-assay precision. Assays were performed by at least three technicians.

	Intra-Assay Precision		Inter-Assay Precision	
Sample	1	2	1	2
n	20	20	10	10
Mean (pg/mL)	143	871	157	990
Standard deviation	6.17	19.4	15.0	57.2
CV (%)	4.3	2.2	9.6	5.8

RECOVERY

The recovery of human PD-1 spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	114	91-131%
Lysis buffer (n=1)	117	94-133%
Serum (n=2)	75	67-82%
EDTA plasma (n=2)	104	91-122%
Heparin plasma (n=2)	85	71-103%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human PD-1 were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=5)	Cell lysates (n=5)	Serum (n=2)	EDTA plasma (n=2)	Heparin plasma (n=2)
1:2	Average % of Expected	87	97	89	89	90
	Range (%)	81-93	86-102	82-96	89-90	84-96
1:4	Average % of Expected	79	95	101	90	92
	Range (%)	68-95	86-106	87-115	84-95	84-100
1:8	Average % of Expected	81	97	113	91	105
	Range (%)	73-94	86-105	99-127	84-99	97-114
1:16	Average % of Expected	70	93	117	93	106
	Range (%)	70-70	89-101	116-117	88-99	106-106

SENSITIVITY

Sixteen assays were evaluated and the minimum detectable dose (MDD) of human PD-1 ranged from 1.75-9.86 pg/mL. The mean MDD was 4.59 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 HEK293-expressed recombinant human PD-1 produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma- Samples from apparently healthy volunteers were evaluated for the presence of human PD-1 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=10)	151	80-314	81.4
EDTA plasma (n=10)	188	117-321	71.8
Heparin plasma (n=0)	188	104-373	93.1

Cell Culture:

Human peripheral blood mononuclear cells (PBMCs) were cultured at 3 x 10^6 cells/mL in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin and were either left untreated or treated with 10 ug/mL PHA-L and 20 ng/mL of recombinant human (rh) IL-2 for 5 days. After 5 days, cells were then treated with 10 ng/mL PMA and 500 ng/mL Ionomycin for an additional 24 hours before harvesting conditioned media.

$CD4^+$ T cells were isolated from PBMCs using MagCellect™ Human $CD4^+$ T Cell Isolation Kit (R&D Systems®, Catalog # MAGH102). For Cloudz™ stimulation, $CD4^+$ T cells were cultured at 5 x 10^5/mL using ExCellerate™ Human T Cell Expansion Media, Xeno-Free (R&D Systems, Catalog # CCM030) for the duration. T cells were treated with 25 µL of Cloudz CD3/28 particles per mL of culture media for 5 days. Cell conditioned media was collected by centrifugation and stored at ≤ -20 °C until assayed. After centrifugation, the remaining cells and Cloudz particles were resuspended with 1X Release Buffer. After dissolution of Cloudz particles, cells were lysed as indicated on the next page.

SAMPLE VALUES *CONTINUED*

Cell Supernates - Aliquots of the cell culture supernates were assayed for levels of human PD-1.

Cell Lysates - Untreated or treated cells were washed with PBS and solubilized in Lysis Buffer 17 (R&D Systems®, Catalog # 895943) supplemented with protease inhibitors using 3-5 times the pellet volume and put on ice for 15 minutes. Tubes were centrifuged at 14,000 x g for 5 minutes to remove insoluble material. The remaining whole cell extract was removed, aliquoted into a clean test tube, and stored at ≤ -20 °C. Whole cell extract protein concentration was quantified using a total protein assay. 10 µg of the cell lysate was removed and assayed for human PD-1.

Condition	Cell culture supernates (pg/mL)	Lysates (pg/mL)
Stimulated $CD4^+$ T cells CD3/28	642	580
Unstimulated PBMC	103	124
Stimulated PBMC	433	398

SPECIFICITY

This assay recognizes natural and recombinant human PD-1.

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 PD-1 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

B7-1
B7-2
B7-H1
B7-H2
B7-H3
B7-H4
B7-H6
B7-H7
PD-L2

Other recombinants:

canine PD-1
mouse PD-1
porcine PD-1
rat PD-1

Recombinant cynomolgus monkey cross reacts 18.3% and interferes at 313 pg/mL.

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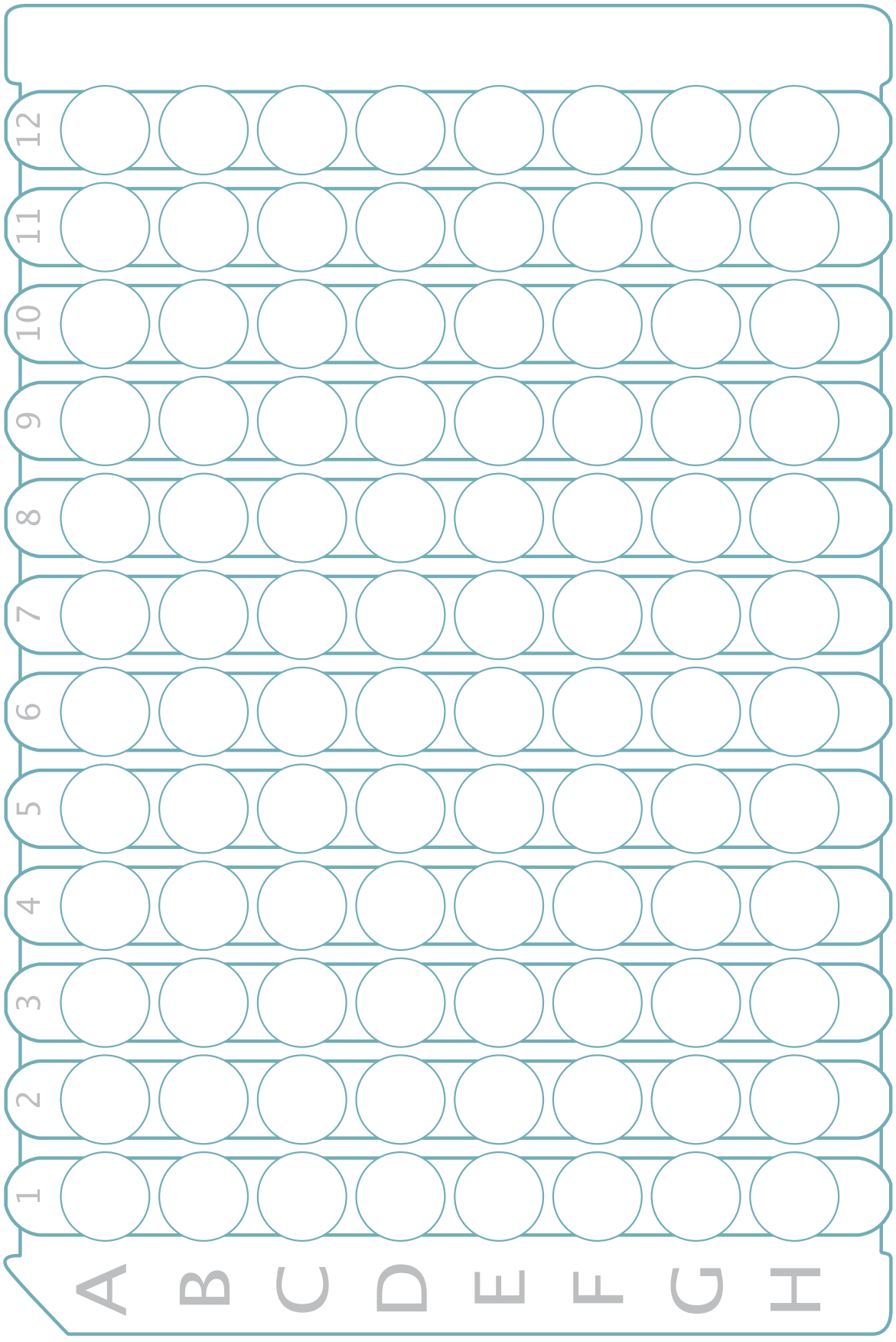
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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