Quantikine® ELISA

Human Nectin-4 Immunoassay

Catalog Number DNEC40

For the quantitative determination of human Nectin-4 concentrations in cell culture supernates, cell lysates, serum, plasma, and urine.

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

Nectin-4, also known as poliovirus receptor-related protein 4 (PVRL4), is an approximately 66 kDa transmembrane adhesion protein that localizes to epithelial adherens junctions (1, 2). Mutations in Nectin-4 are associated with ectodermal dysplasia-syndactyly syndrome (EDSS), a disorder of hair, tooth, and skin abnormalities (3). Human Nectin-4 consists of a 318 amino acid (aa) extracellular domain (ECD) with three immunoglobulin-like domains, a 21 aa transmembrane segment, and a 140 aa cytoplasmic domain (4). Within the ECD, human Nectin-4 shares 91% aa sequence identity with mouse and rat Nectin-4. Nectin-4 interacts homophilically as well as heterophilically with Nectin-1 and Integrin β4 (4-6). It is expressed in trachea, prostate, lung, stomach, keratinocytes, hair bulbs, and vascular endothelial cells in the placenta (3, 4). It is upregulated on some breast, ovarian, lung, and colon cancer cells (7-10). Nectin-4 contributes to the anchorage-independent growth and increased invasiveness of tumor cells (6, 10). A 43 kDa fragment of its ECD is shed from the cell surface by TACE/ADAM17 mediated cleavage (11). This fragment circulates in the serum of breast, serous ovarian, and NSCLC lung cancer patients (7, 8, 10, 11). In addition, Nectin-4 is required for the measles virus to gain cellular entry and to spread between cells of the epithelium (9, 12).

The Quantikine Human Nectin-4 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Nectin-4 in cell culture supernates, cell lysates, serum, plasma, and urine. It contains NS0-expressed recombinant human Nectin-4 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Nectin-4 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 naturally occurring human Nectin-4.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Nectin-4 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Nectin-4 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Nectin-4 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 Nectin-4 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.
- Samples, controls, and standards must be pipetted within 15 minutes.
- If samples generate values higher than the highest standard, further 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.
- 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.

PRECAUTIONS

Nectin-4 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® 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. Please refer to the MSDS on our website prior to use.

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
Human Nectin-4 Microplate	894621	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Nectin-4.	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 Nectin-4 Conjugate	894622	21 mL of a polyclonal antibody specific for human Nectin-4 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Nectin-4 Standard	894623	Recombinant human Nectin-4 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-40	895513	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *For serum/plasma samples. Used diluted 1:4 in this assay.*	
Calibrator Diluent RD5-27	895395	21 mL of a buffered protein base with preservatives. *For cell culture supernate/ cell lysate/urine samples. Used undiluted in this assay.*	
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.

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 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.
- Human Nectin-4 Controls (optional; available from R&D Systems).

SUPPLIES REQUIRED FOR CELL LYSATE SAMPLES

- Cell Lysis Buffer 2 (R&D Systems, Catalog # 895347)
- PBS

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 Cell Lysis Procedure.

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.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Repeated freeze-thaw cycles have shown significantly lower urine sample readings.*

SAMPLE PREPARATION

Cell lysate samples require a 2-fold dilution. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5-27.

Urine samples require a 4-fold dilution. A suggested 4-fold dilution is 40 µL of sample + 120 µL of Calibrator Diluent RD5-27.

CELL LYSIS PROCEDURE

Use the following procedure for the preparation of cell lysate samples.

1. Wash cells three times in cold PBS.
2. Resuspend cells at 1 x 10^7 cells/mL in Cell Lysis Buffer 2.
3. Incubate with gentle agitation for up to 60 minutes at room temperature.
4. Centrifuge at 8000 x g for 10 minutes to remove cell debris.
5. Assay immediately or aliquot the lysis supernates and store at ≤ -70 °C until ready for use.

REAGENT PREPARATION

The Human Nectin-4 Conjugate must remain at 2-8 °C during use.
Bring all reagents to room temperature before use.

Note: *Nectin-4 is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

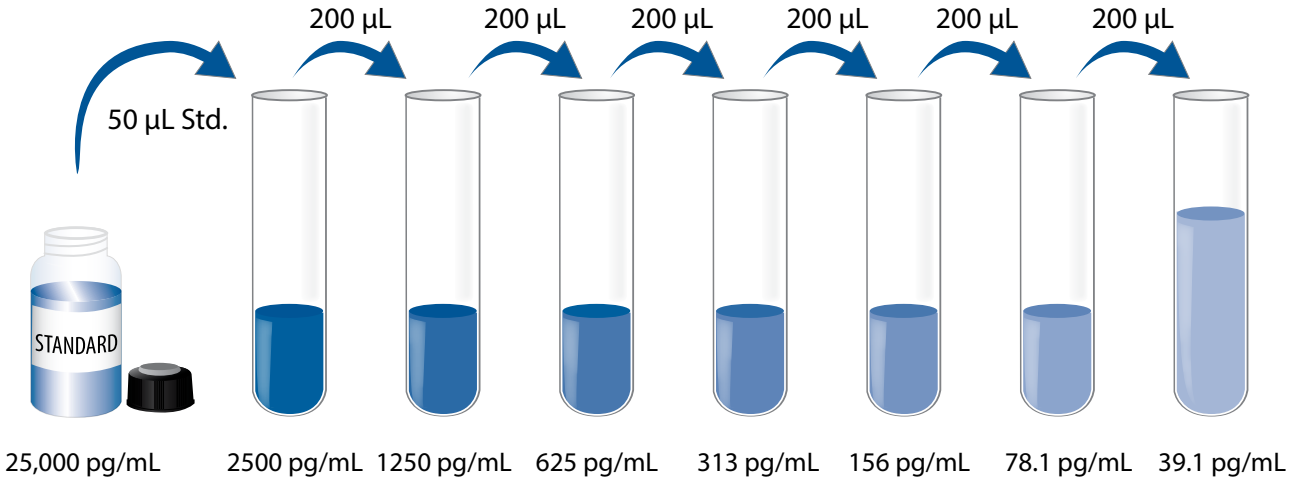
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD5-26 (diluted 1:4) - **For serum/plasma samples only.** Add 20 mL of Calibrator Diluent RD5-26 Concentrate to 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-26 (diluted 1:4).

Human Nectin-4 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Nectin-4 Standard with deionized or distilled water. This reconstitution produces a stock solution of 25,000 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 450 µL of Calibrator Diluent RD5-27 (*for cell culture supernate/cell lysate/urine samples*) or Calibrator Diluent RD5-26 (diluted 1:4) (*for serum/plasma samples*) into the 2500 pg/mL tube. Pipette 200 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 2500 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

The Conjugate must remain at 2-8 °C. Bring all other reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *Nectin-4 is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 Assay Diluent RD1-40 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 2-8 °C.** A plate layout is provided to record standards and samples assayed.

   Note: *Samples, controls, and standards must be pipetted within 15 minutes.*
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 **cold** Human Nectin-4 Conjugate to each well. Cover with a new adhesive strip. **Incubate for 2 hours at 2-8 °C.**
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 on the benchtop. **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.

*Samples may require dilution. See the 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.).

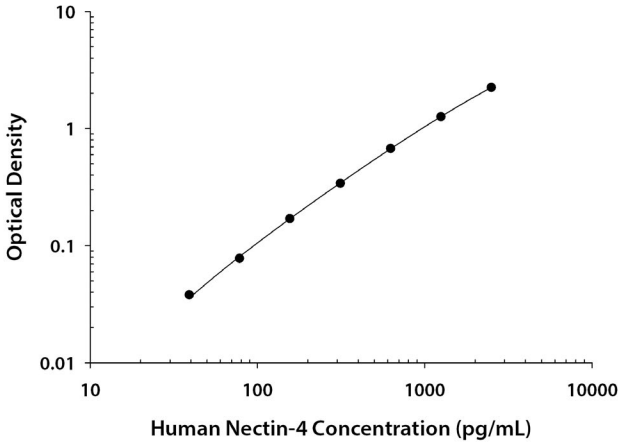
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best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

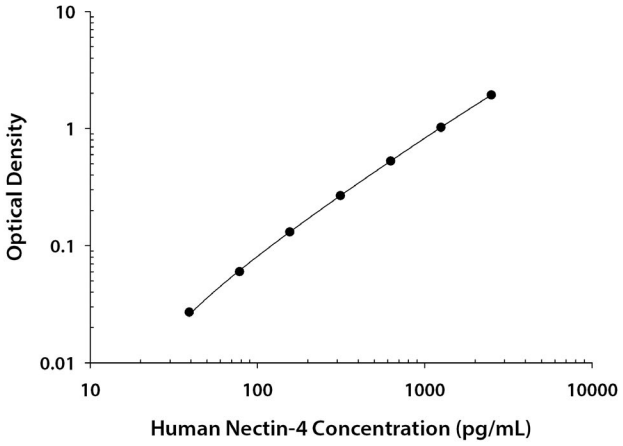
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/CELL LYSATE/URINE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.032 0.033	0.033	—
39.1	0.071 0.071	0.071	0.038
78.1	0.110 0.112	0.111	0.078
156	0.202 0.203	0.203	0.170
313	0.369 0.378	0.374	0.341
625	0.691 0.726	0.709	0.676
1250	1.281 1.311	1.296	1.263
2500	2.249 2.301	2.275	2.242

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.033 0.034	0.034	—
39.1	0.060 0.061	0.061	0.027
78.1	0.091 0.097	0.094	0.060
156	0.163 0.166	0.165	0.131
313	0.299 0.302	0.301	0.267
625	0.556 0.570	0.563	0.529
1250	1.056 1.058	1.057	1.023
2500	1.938 1.996	1.967	1.933

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

CELL CULTURE SUPERNATE/CELL LYSATE/URINE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	163	425	1365	194	474	1440
Standard deviation	5.56	13.9	32.7	15.8	27.9	65.8
CV (%)	3.4	3.3	2.4	8.1	5.9	4.6

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)	232	603	1867	245	595	1801
Standard deviation	7.19	37.7	40.1	18.9	31.9	82.3
CV (%)	3.1	6.3	2.1	7.7	5.4	4.6

RECOVERY

The recovery of human Nectin-4 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	100	94-110%
Serum (n=4)	90	80-101%
EDTA plasma (n=4)	89	80-109%
Heparin plasma (n=4)	91	82-99%
Urine* (n=4)	100	93-108%

*Samples were diluted prior to assay.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Nectin-4 were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Cell lysates* (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine* (n=4)
1:2	Average % of Expected	103	96	98	94	93	104
	Range (%)	97-113	87-105	95-101	90-96	87-96	99-111
1:4	Average % of Expected	100	98	105	103	102	110
	Range (%)	91-114	88-113	102-108	95-107	94-110	103-116
1:8	Average % of Expected	100	96	113	104	109	106
	Range (%)	90-117	85-110	106-120	94-113	102-119	101-113
1:16	Average % of Expected	92	101	108	107	106	113
	Range (%)	86-96	98-106	102-115	97-114	94-117	103-119

*Samples were diluted prior to assay.

SENSITIVITY

Forty-four assays were evaluated and the minimum detectable dose (MDD) of human Nectin-4 ranged from 2.95-16.6 pg/mL. The mean MDD was 6.56 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 Nectin-4 manufactured at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human Nectin-4 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=36)	236	122-348	52.8
EDTA plasma (n=36)	215	104-321	51.7
Heparin plasma (n=36)	229	112-356	52.6
Urine* (n=10)	2450	999-6120	1519

*Samples were subjected to only one freeze-thaw cycle prior to assay.

Cell Culture Supernates/Cell Lysates:
ZR-75 human breast cancer cells were cultured in DMEM supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 100 µg/mL streptomycin sulfate, and grown until confluent.

MDA-MB-453 human breast cancer cells were cultured in RPMI supplemented with 10% fetal bovine serum and 2 mM L-glutamine, and grown until confluent.

OVCAR-3 human ovarian carcinoma cells were cultured in RPMI supplemented with 20% fetal bovine serum, 10 µg/mL insulin, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate, and grown until confluent.

An aliquot of each cell culture supernate was removed and assayed for human Nectin-4. The cells were lysed according to the Cell Lysis Procedure and assayed for human Nectin-4. Cell lysate was normalized to total protein concentration.

Cell Line	Cell Culture Supernate Values (pg/mL)	Cell Lysate Values (pg/mg)
ZR-75	2232	976
MDA-MB-453	1466	7685
OVCAR-3	291	969

SPECIFICITY

This assay recognizes natural and recombinant human Nectin-4.

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 Nectin-4 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

CD155/PVR
IGSF4A/SynCAM1
IGSF4B/SynCAM3
IGSF4C/SynCAM4
IGSF4D/SynCAM2
Nectin-1
Nectin-2
Nectin-3

Recombinant mouse:

Nectin-4

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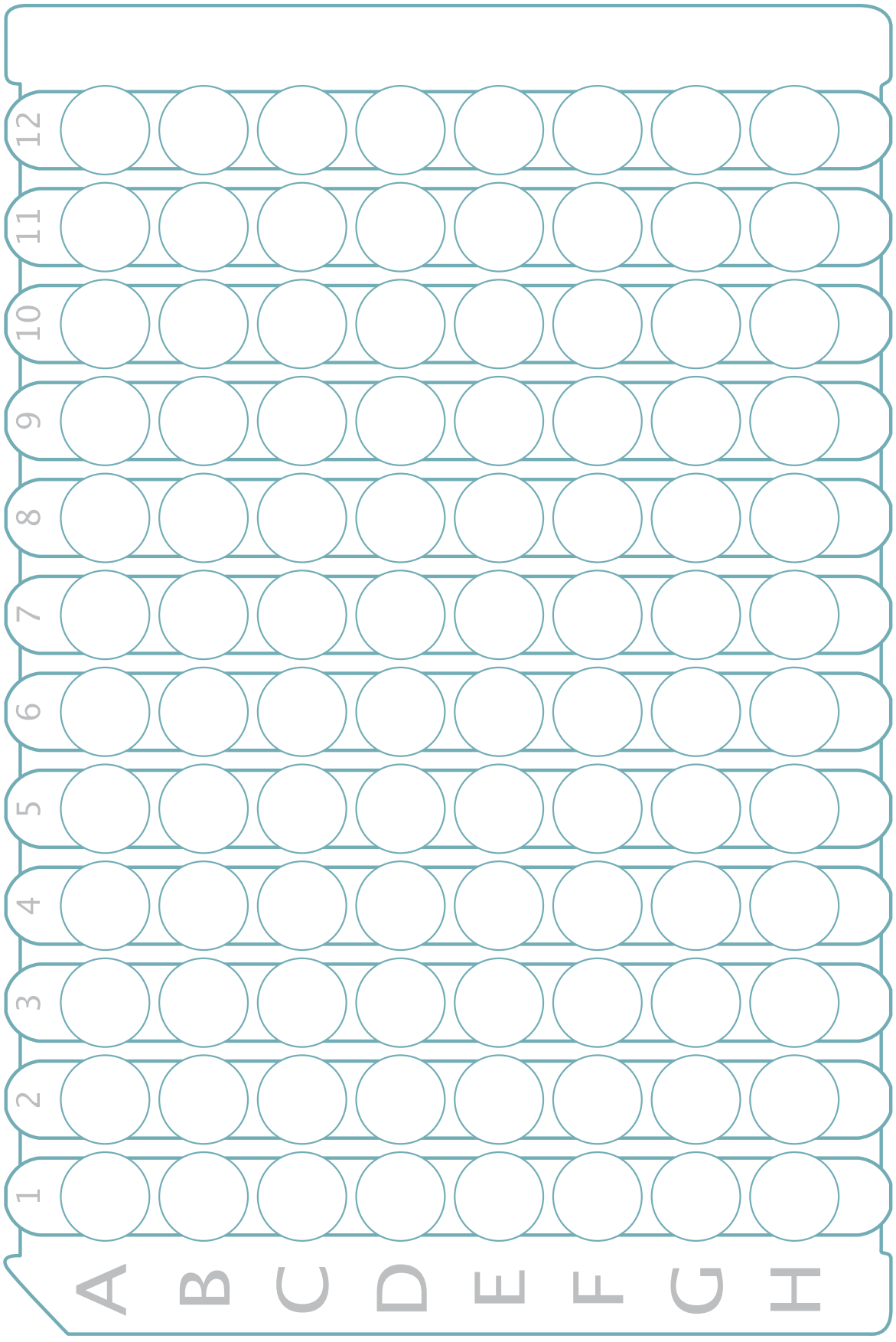
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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