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Saliva Sensitive

Quantikine™ QuicKit™ ELISA

Human Lipocalin-2/NGAL Immunoassay

Catalog Number QK1757

For the quantitative determination of human Lipocalin-2 concentrations in cell culture supernates, serum, heparin plasma, saliva, 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

Members of the Lipocalin family have limited sequence identity, but share a highly conserved fold with an eight-stranded anti-parallel β barrel motif that encloses an internal ligand-binding site (1, 2). They are known for their actions as transporters that carry small hydrophobic molecules such as steroid hormones, vitamins, odorants, and metabolic products (1-4). Lipocalin-2, also known as Neutrophil Gelatinase-associated Lipocalin (NGAL) or Siderocalin, was originally identified as a component of neutrophil granules (5). Since then, its expression has been observed in most tissues, and its synthesis is induced in epithelial cells during inflammation (4, 6). Lipocalin-2 has been implicated in a variety of cellular processes including the innate immune response, differentiation, tumorigenesis, and cell survival (4, 7-9). It is a 25 kDa protein existing in monomeric, homodimeric, and heterodimeric forms, the latter in association with human matrix metalloproteinase 9 (MMP-9) (5). Its association with MMP-9 may modulate protease activity by protecting MMP-9 from degradation (10). The mouse ortholog (also known as 24p3) shares 62% sequence identity at the amino acid level (4, 5).

The functions of Lipocalin-2 continue to be elucidated. Studies indicate that it binds bacterial catecholate siderophores bound to ferric ions (9, 11). This suggests that Lipocalin-2 may act as a bacteriostatic agent by binding bacterial siderophores and limiting bacterial iron supply. This is supported by the observation that mouse Lipocalin-2 is induced in immune cells following Toll-like receptor activation, and Lipocalin-2 mouse knockouts exhibit decreased ability to counter bacterial infection (9, 12). Lipocalin-2 may also regulate iron uptake into mammalian cells (8). In the kidney, Lipocalin-2-mediated iron trafficking may be involved in both development and protection from renal injury (8, 13-15). Megalin, a member of the LDL receptor family, and 24p3 R have been reported as endocytic receptors for Lipocalin-2 (16, 17). It should be noted that the effects of Lipocalin-2 on cells might be context-dependent. For instance, it has been shown to act as both a survival factor and a pro-apoptotic factor, and its induction by pro-inflammatory cytokines may vary between mouse and human (17-19).

Lipocalin-2 has been associated with several pathological processes. For instance, it is upregulated in psoriatic skin in comparison to uninvolved control skin, and Lipocalin-2 suppresses red blood cell production in models of anemia (20, 21). Lipocalin-2 is elevated in patients with severe acute respiratory syndrome (SARS), and may act as a biomarker for acute renal injury (15, 22). It has been associated with several tumor types as well, including breast, ovarian, colorectal, and pancreatic cancers (23-26). Its function in cancer is unclear, although the invasive and metastatic behavior of tumor cells is suppressed by Lipocalin-2 in models of breast and colon cancer (27, 28).

The Quantikine™ QuicKit™ Human Lipocalin-2/NGAL Immunoassay is a one step, 80-minute solid phase ELISA designed to measure human Lipocalin-2 levels in cell culture supernates, serum, heparin plasma, saliva, and urine. It contains recombinant human Lipocalin-2 and antibodies raised against the recombinant protein. Results obtained using natural human Lipocalin-2 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 Lipocalin-2.

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 Lipocalin-2. After washing away any unbound substances, a substrate solution is added to the wells and color develops in proportion to the amount of Lipocalin-2 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 with those 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 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.

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
QuickKit™ 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 Lipocalin-2 Standard	899194	2 vials of recombinant human Lipocalin-2 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 Lipocalin-2 Capture Ab Concentrate	899192	Lyophilized tagged monoclonal antibody specific for human Lipocalin-2.	May be stored for up to 1 month at 2-8 °C.*
Human Lipocalin-2 Detection Ab Concentrate	899193	400 µL of a monoclonal antibody specific for human Lipocalin-2 conjugated to horseradish peroxidase with preservatives.	
Assay Diluent RD1-52	895343	11 mL of a buffered protein base with blue dye and preservatives.	
Calibrator Diluent RD5-3	895436	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 preservatives. *May turn yellow over time.*	
TMB ELISA Substrate	642736	12 mL of a TMB ELISA substrate.	
ELISA Stop Solution	642827	12 mL of an acid solution.	
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
- 500 mL graduated cylinder
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards
- Human Lipocalin-2 Controls (optional; R&D Systems™, Catalog # QC286)

PRECAUTIONS

Lipocalin-2 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 a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

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.

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 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: *EDTA and Citrate plasma have not been validated for use in this assay.*

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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

SAMPLE PREPARATION

Serum and plasma samples require a 20-fold dilution due to matrix effect. A suggested 20-fold dilution is 10 µL of sample + 190 µL of Calibrator Diluent RD5-3.

Urine samples require a 2-fold dilution due to matrix effect. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5-3.

Saliva samples require a 50-fold dilution due to high endogenous levels. A suggested 50-fold dilution is 10 µL of sample + 490 µL of Calibrator Diluent RD5-3.

Cell culture supernates can be run neat. Multiple dilutions are recommended for unknown samples.

REAGENT PREPARATION

Note: *High concentrations of Lipocalin-2 are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

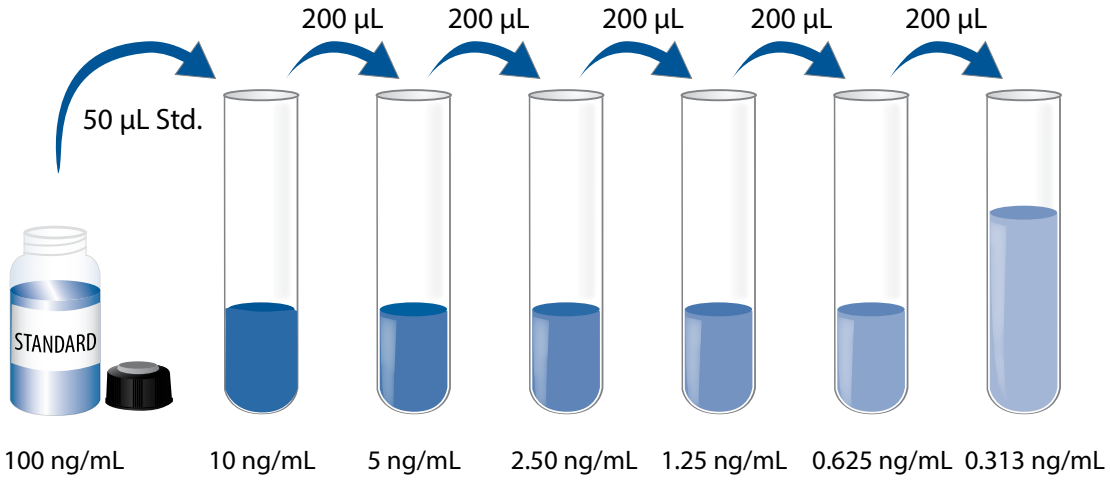
Human Lipocalin-2 Capture Ab Concentrate - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Lipocalin-2 Capture Ab Concentrate with Assay Diluent RD1-52. This reconstitution produces a 20X Capture Antibody stock. Allow the capture antibody to sit for a minimum of 5 minutes with gentle agitation prior to diluting. Once reconstituted, the 20X capture antibody 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 RD1-52. For a full plate, add 300 µL of reconstituted Human Lipocalin-2 Capture Ab stock and 300 µL of Human Lipocalin-2 Detection Ab Concentrate to 5.4 mL of Assay Diluent RD1-52 to get 6 mL of Human Lipocalin-2 Antibody Cocktail.

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.

Human Lipocalin-2 Standard - **Refer to vial label for reconstitution volume.** Reconstitute the Human Lipocalin-2 Standard with distilled or deionized water. This reconstitution produces a stock solution of 100 ng/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 RD5-3 into the 10 ng/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 10 ng/mL standard serves as the high standard. Calibrator Diluent RD5-3 serves as the zero standard (0 ng/mL).



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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.

Note: *High concentrations of Lipocalin-2 are 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 standard, control, or sample* per well. A plate layout is provided to record standards and samples assayed.
4. Add 50 µL Antibody Cocktail to each well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal microplate orbital shaker 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 100 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

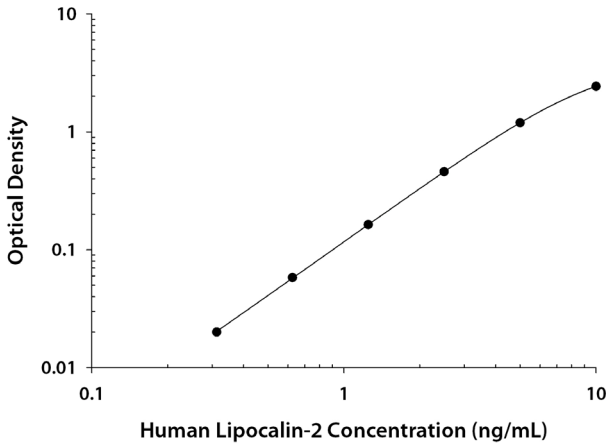
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Lipocalin-2 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.



(ng/mL)	O.D.	Average	Corrected
0	0.003 0.006	0.005	—
0.313	0.024 0.026	0.025	0.020
0.625	0.062 0.063	0.063	0.058
1.25	0.167 0.169	0.168	0.163
2.50	0.458 0.470	0.464	0.459
5	1.196 1.204	1.200	1.195
10	2.425 2.447	2.436	2.431

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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 (ng/mL)	1.01	6.05	1.11	6.46
Standard deviation	0.025	0.261	0.058	0.379
CV (%)	2.5	4.3	5.2	5.9

RECOVERY

The recovery of human Lipocalin-2 spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	108	100-115%
Urine (n=2)	102	89-110%

LINEARITY

To assess linearity of the assay, samples containing and/or spiked with high concentrations of human Lipocalin-2 in various matrices were diluted with the calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Serum* (n=2)	Heparin plasma* (n=2)	Saliva* (n=2)	Urine* (n=2)
1:2	Average % of Expected	96	114	105	102	100
	Range (%)	80-116	112-116	104-106	100-104	98-102
1:4	Average % of Expected	94	124	110	104	101
	Range (%)	77-122	120-127	108-112	102-105	98-105
1:8	Average % of Expected	92	124	113	106	105
	Range (%)	72-124	123-124	111-115	105-108	99-110
1:16	Average % of Expected	96	115	107	100	98
	Range (%)	72-120	111-118	104-110	98-101	89-108

*Samples were diluted prior to assay.

SENSITIVITY

Eighteen assays were evaluated and the minimum detectable dose (MDD) of human Lipocalin-2 ranged from 0.008-0.033 ng/mL. The mean MDD was 0.016 ng/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 recombinant human Lipocalin-2 produced at R&D Systems™.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human Lipocalin-2 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=10)	79.3	50.9-129	25.7
Heparin plasma (n=10)	64.3	46.3-94.9	18.3
Saliva (n=10)	387	62.1-734	217
Urine (n=10)	11.3	1.18-27.9	9.37

Cell Culture Supernates:
Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 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 supernates were removed and assayed for levels of human Lipocalin-2.

Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Unstimulated	2.59	5.18
Stimulated	3.28	4.58

A431 human epithelial carcinoma cells were grown to confluency in DMEM (high glucose) with 10% fetal bovine serum. Aliquots of the cell culture supernate was removed, assayed for human Lipocalin-2, and measured 26.9 ng/mL.

Neutrophils were cultured at 1 x 10^7 cells/mL in Hank's media and stimulated with 50 ng/mL PMA for 30 minutes. Aliquots of the cell culture supernate was removed, assayed for human Lipocalin-2, and measured 89.9 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Lipocalin-2.

The factors listed below were prepared at 100 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 100 ng/mL in a low level recombinant human Lipocalin-2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

COX-2
Lipocalin-1
Megalin-C3
Megalin-C4
MMP-9

Other recombinants:

mouse Lipocalin-2
rat NGAL

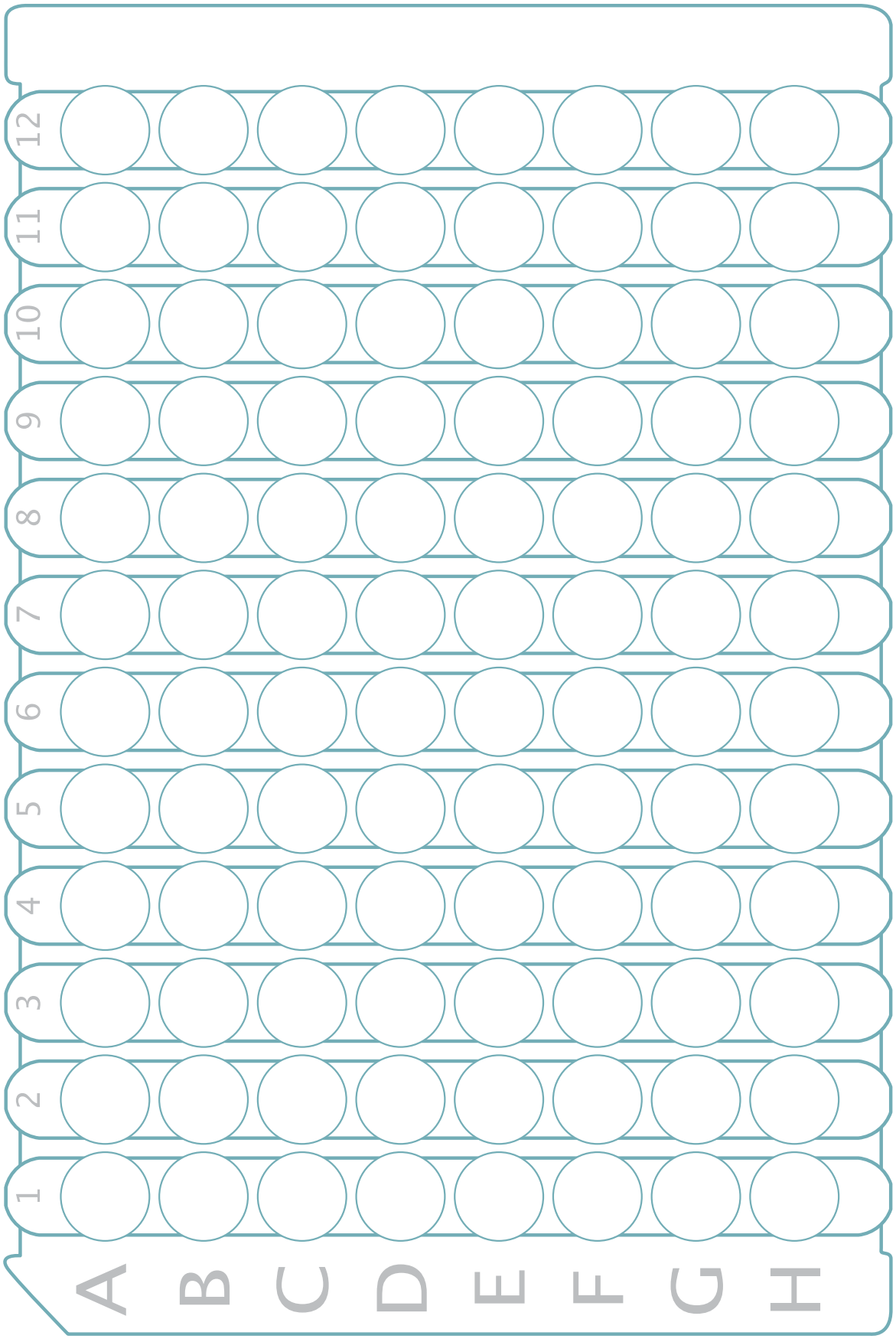
Recombinant human MMP-9/Lipocalin-2 Complex cross-reacts approximately 11.8% and interferes at concentrations > 5 ng/mL in this assay.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES

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