Quantikine® ELISA

Human Lipocalin-2/NGAL Immunoassay

Catalog Number DLCN20
SLCN20
PDLCN20

For the quantitative determination of human Lipocalin-2/NGAL concentrations in cell culture supernates, serum, 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 Human Lipocalin-2/NGAL Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Lipocalin-2 in cell culture supernates, serum, plasma, saliva, and urine. It contains NS0-expressed recombinant human Lipocalin-2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Lipocalin-2 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 Lipocalin-2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Lipocalin-2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Lipocalin-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Lipocalin-2 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 Lipocalin-2 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.
- 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # DLCN20	CATALOG # SLCN20	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Lipocalin-2 Microplate	893023	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Lipocalin-2.	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.*
Human Lipocalin-2 Conjugate	893024	1 vial	6 vials	21 mL/vial of a monoclonal antibody specific for human Lipocalin-2 conjugated to horseradish peroxidase with preservatives. *The Lipocalin-2 Conjugate must remain at 2-8 °C during use.*	May be stored for up to1 month at 2-8 °C.*
Human Lipocalin-2 Standard	893025	1 vial	6 vials	100 ng/vial of recombinant human Lipocalin-2 in a buffered protein base with preservatives; lyophilized.	
Assay Diluent RD1-52	895343	1 vial	6 vials	11 mL/vial of a buffered protein base with preservatives. *May contain a precipitate. Mix well before and during use.*	
Calibrator Diluent RD5-24 Concentrate	895325	1 vial	6 vials	21 mL/vial of a concentrated solution containing a buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

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

DLCN20 contains sufficient materials to run an ELISA on one 96 well plate.
SLCN20 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PDLCN20). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

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.
- 2-8 °C incubator.
- Collection device for saliva samples that has no protein binding or filtering capabilities such as a Salivette® or equivalent.
- Test tubes for dilution of standards and samples.
- Human Lipocalin-2 Controls (optional; R&D Systems, Catalog # QC115).

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 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.

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: *Citrate plasma has not been validated for use in this assay.*
EDTA plasma is not recommended for use in this assay due to its chelating properties.

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

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. A suggested 20-fold dilution is 20 µL of sample + 380 µL of Calibrator Diluent RD5-24 (diluted 1:5).

Saliva samples require a minimum 50-fold dilution. A suggested 50-fold dilution is 20 µL of sample + 980 µL of Calibrator Diluent RD5-24 (diluted 1:5).

Urine and cell culture supernate samples may require dilution.

REAGENT PREPARATION

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

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.*

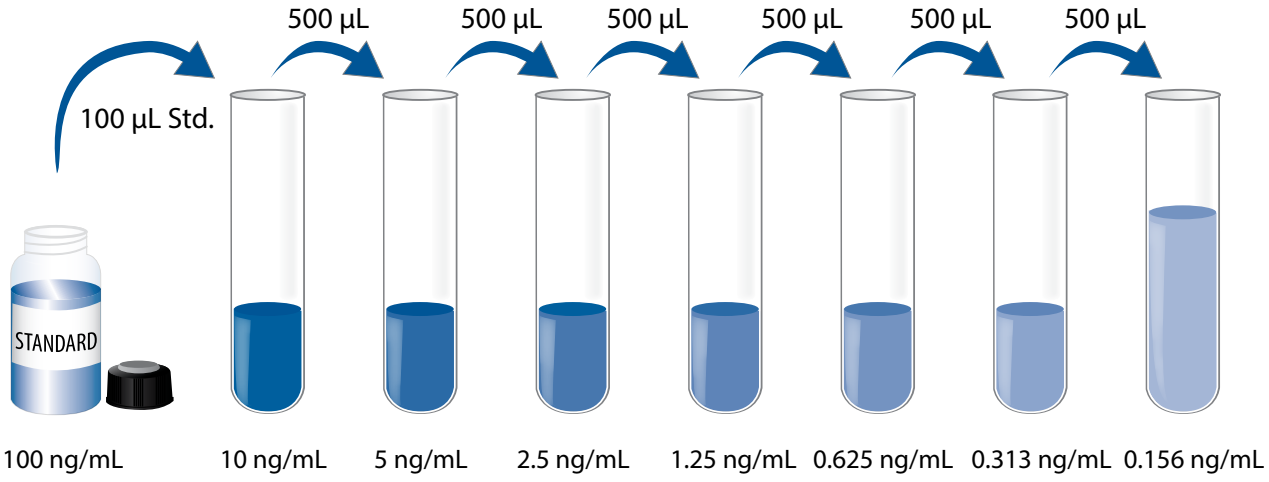
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD5-24 (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5-24 Concentrate to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5-24 (diluted 1:5).

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.

Human Lipocalin-2 Standard - Reconstitute the Human Lipocalin-2 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 100 ng/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 900 µL of Calibrator Diluent RD5-24 (diluted 1:5) into the 10 ng/mL tube. Pipette 500 µL of Calibrator Diluent RD5-24 (diluted 1:5) 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 RD5-24 (diluted 1:5) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

The Human Lipocalin-2 Conjugate must remain at 2-8 °C during use. Bring all other reagents and samples to room temperature before use. It is recommended that all samples and standards 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 100 µL of Assay Diluent RD1-52 to each well. *Assay Diluent RD1-52 may contain a precipitate. Mix well before and during use.*
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.**
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 Lipocalin-2 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. 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 Sample Preparation section.

CALCULATION OF RESULTS

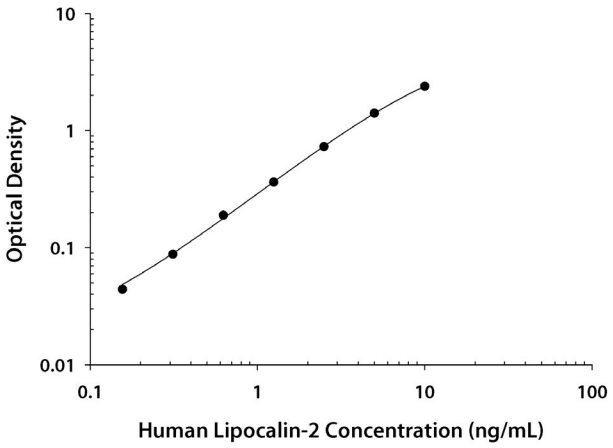
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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	0.011 0.011	0.011	—
0.156	0.054 0.055	0.055	0.044
0.313	0.095 0.102	0.099	0.088
0.625	0.199 0.200	0.200	0.189
1.25	0.372 0.375	0.374	0.363
2.5	0.732 0.749	0.741	0.730
5	1.380 1.451	1.416	1.405
10	2.356 2.443	2.400	2.389

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

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	1.14	3.45	7.54	1.05	3.37	6.92
Standard deviation	0.041	0.107	0.334	0.083	0.204	0.387
CV (%)	3.6	3.1	4.4	7.9	6.1	5.6

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	93	88-101%
Urine* (n=4)	90	84-97%

*2 of 4 samples were diluted prior to spiking.

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human Lipocalin-2 were serially diluted with the Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Serum* (n=4)	Heparin plasma* (n=4)	Saliva* (n=4)	Urine* (n=4)
1:2	Average % of Expected	104	104	105	100	101
	Range (%)	96-108	102-106	102-108	97-101	99-103
1:4	Average % of Expected	99	107	113	101	99
	Range (%)	90-104	104-110	106-121	98-104	97-101
1:8	Average % of Expected	100	111	113	100	101
	Range (%)	91-106	107-116	108-119	97-103	95-108
1:16	Average % of Expected	102	109	114	100	103
	Range (%)	93-108	103-113	110-118	96-102	99-109

*Samples were diluted prior to assay.

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of human Lipocalin-2 ranged from 0.003-0.040 ng/mL. The mean MDD was 0.012 ng/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 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=35)	119	42-177	35.6
Heparin plasma (n=35)	94	36-149	28.7
Saliva (n=9)	320	96-881	238
Urine (n=19)	9.94	0.40-72	17.1

Cell Culture Supernates:
Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal calf serum, 50 µM β-mercaptoethanol, 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 natural human Lipocalin-2.

Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Unstimulated	7.73	7.73
Stimulated	14.3	18.5

A431 human epithelial carcinoma cells were grown to confluency in DMEM (high glucose) with 10% fetal calf serum. An aliquot of the cell culture supernate was removed, assayed for levels of natural human Lipocalin-2, and measured 130 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. An aliquot of the cell culture supernate was removed, assayed for levels of natural human Lipocalin-2, and measured 699 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Lipocalin-2.

The factors listed below were prepared at 200 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range recombinant human Lipocalin-2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

COX-2
Lipocalin-1
MMP-9

Recombinant mouse:

Lipocalin-2

Recombinant human MMP-9/NGAL Complex cross-reacts approximately 0.3% in this assay.

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