BioLegend®

Enabling Legendary Discovery™

LEGEND MAX™

ELISA Kit

Human NGAL (Lipocalin-2)

Cat. No. 443407

ELISA Kit for Accurate Quantitation of Human NGAL from Cell Culture Supernatant, Serum, Plasma, Urine, Saliva and Other Biological Fluids

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It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

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Introduction:

Neutrophil Gelatinase-Associated Lipocalin (NGAL), also known as Lipocalin-2, Siderocalin, 24p3, Uterocalin or Neu-related lipocalin, is a member of the lipocalin family. It is a secreted protein (25kD), which captures bacterial siderophores that bind iron with high affinity. NGAL predominantly exists in monomeric form with a small portion in homodimeric and homotrimeric forms. It can also form a heterodimer with MMP-9 and a heterotrimer with MMP-9 and TIMP-1. Human NGAL shares 62% amino acid sequence identity with mouse NGAL.

The function of NGAL is diverse and continues to be studied. NGAL acts as a potent anti-bacterial agent via siderophore-mediated iron depletion. NGAL knockout mice exhibit decreased ability to combat bacterial infection. NGAL has been used as an early predictive and prognostic biomarker for acute and chronic kidney injuries. It also has been associated with breast, lung, colon and pancreatic cancer, having diverse, context-dependent effects during tumor development. This may involve cell growth, survival, migration, invasion and angiogenesis. When NGAL form a complex with MMP-9, it protects MMP-9 from degradation, thereby preserving MMP-9's enzymatic activity, which has been involved in various cancers.

The LEGEND MAX™ Human NGAL ELISA kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with a mouse monoclonal anti-human NGAL antibody. The Detection Antibody is a biotinylated mouse monoclonal anti-human NGAL antibody. This kit is specifically designed for the accurate quantitation of human NGAL from cell culture supernatant, serum, plasma, urine, saliva, and other biological fluids. This kit is analytically validated with ready-to-use reagents.

Materials Provided:

Description	Quantity	Volume (per bottle)	Part #
Anti-Human NGAL Pre-coated 96 well Strip Microplate	1 plate		76197
Human NGAL Detection Antibody	1 bottle	12 mL	76198
Human NGAL Standard	1 vial	lyophilized	76200
Avidin-HRP	1 bottle	12 mL	77897
Assay Buffer B	1 bottle	25 mL	79128
Wash Buffer (20X)	1 bottle	50 mL	78233
Substrate Solution D	1 bottle	12 mL	78115
Stop Solution	1 bottle	12 mL	79133
Plate Sealers	4 sheets		78101

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Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 µL to 1,000 µL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Plate Shaker
- Polypropylene vials

Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

<table>
<tr><th colspan="2">Opened or Reconstituted Components</th></tr>
<tr><td>Microplate wells</td><td>If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month.</td></tr>
<tr><td>Standard</td><td>The remaining reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Detection Antibody</td><td rowspan="6">Store opened reagents between 2°C and 8°C and use within one month.</td></tr>
<tr><td>Avidin-HRP</td></tr>
<tr><td>Assay Buffer B</td></tr>
<tr><td>Wash Buffer (20X)</td></tr>
<tr><td>Substrate Solution D</td></tr>
<tr><td>Stop Solution</td></tr>
</table>

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution D is harmful if inhaled or ingested. Avoid skin, eye and clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma and other biological fluids in accordance with

NCCLS regulations.

4. Stop Solution contains strong acid. *Wear eye, hand, and face protection.*

5. Before disposing of the plate, rinse it with an excess amount of tap water.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 x *g*. Remove serum layer. *Assay immediately or store serum samples at < -70°C. Avoid repeated freeze-thaw cycles.*

Plasma: Collect blood samples in heparin, citrate or EDTA-containing tubes. Centrifuge for 10 minutes at 1,000 x *g* within 30 minutes of collection. *Assay immediately or store plasma samples at < -70°C. Avoid repeated freeze-thaw cycles*.

Urine: Aseptically collect the first urine of the day (mid-stream) directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at < -70*°C. Avoid repeated freeze-thaw cycles.*

Saliva: Collect saliva samples from passive drool by using a piece of plastic drinking straw (cut into a 2 inch piece) in a small vial. Centrifuge to remove particulate matter, assay immediately or aliquot and store at < -70°C. *Avoid repeated freeze-thaw cycles.*

Reagent and Sample Preparation:

Note: All reagents should be diluted immediately prior to use.

1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and mix until dissolved.

2. Reconstitute the lyophilized Human NGAL Standard by adding the volume of Assay Buffer B to make the 100 ng/mL standard stock solution (Refer to LEGEND MAX Kit Lot-Specific Certificate of Analysis/LEGEND MAX Kit Protocol). Allow the reconstituted standard to sit at room temperature for 15 minutes, then briefly vortex to mix completely.

3. For cell culture supernatant samples, the end user may need to determine the dilution factor needed in a preliminary experiment. If dilutions are necessary, samples should be diluted in the corresponding cell culture medium.

4. A minimum of a 100-fold dilution is recommended for serum or plasma, a

20-fold dilution for urine, and a 2000-fold dilution for saliva. All dilutions should be prepared in Assay Buffer B. For example, 5 µL of serum sample should be added to 495 µL of Assay Buffer B to make a 1:100 dilution.

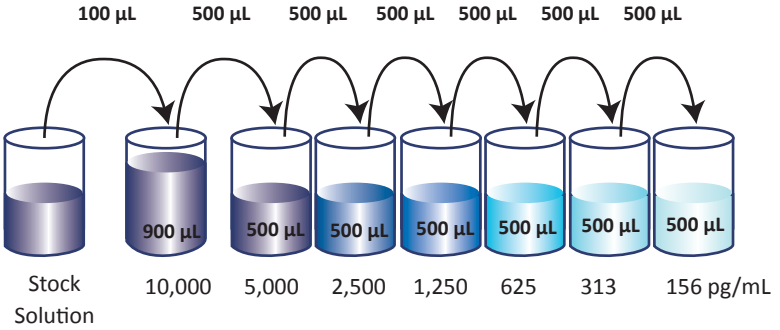
Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit.

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate. A standard curve is required for each assay.

2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.

3. Prepare 1,000 µL of the 10 ng/mL top standard by diluting 100 µL of the standard stock solution in 900 µL of Assay Buffer B. Perform six two-fold serial dilutions of the 10 ng/mL top standard in separate tubes using Assay Buffer B as the diluent. Thus, the human NGAL standard concentrations in the tubes are 10,000 pg/mL, 5,000 pg/mL, 2,500 pg/mL, 1,250 pg/mL, 625 pg/mL, 313 pg/mL and 156 pg/mL, respectively. Assay Buffer B serves as the zero standard (0 pg/mL).



4. For measuring samples:
   a) Add 50 µL of Assay Buffer B to each well that will contain either standards or samples.
   b) Add 50 µL of standard dilutions to the wells for standards. Add 50 µL of samples to the wells for samples.

5. Seal the plate with a Plate Sealer included in the kit and incubate the plate at room temperature for 2 hours while shaking at 200 rpm.

6. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer. Wash the plate with at least 300 µL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping plate upside down

on absorbent paper. All subsequent washes should be performed similarly.

7. Add 100 µL of Human NGAL Detection Antibody solution to each well, seal the plate and incubate at room temperature for 1 hour while shaking.

8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 6.

9. Add 100 µL of Avidin-HRP solution to each well, seal the plate and incubate at room temperature for 30 minutes while shaking.

10. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 6. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

11. Add 100 µL of Substrate Solution D to each well and incubate for 15 minutes in the dark. Wells containing human NGAL should turn blue in color with intensity proportional to concentration. It is not necessary to seal the plate during this step.

12. Stop the reaction by adding 100 µL of Stop Solution to each well. The well color should change from blue to yellow.

13. Read absorbance at 450 nm within 20 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

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Assay Procedure Summary

1. Add 50 µL Assay Buffer B

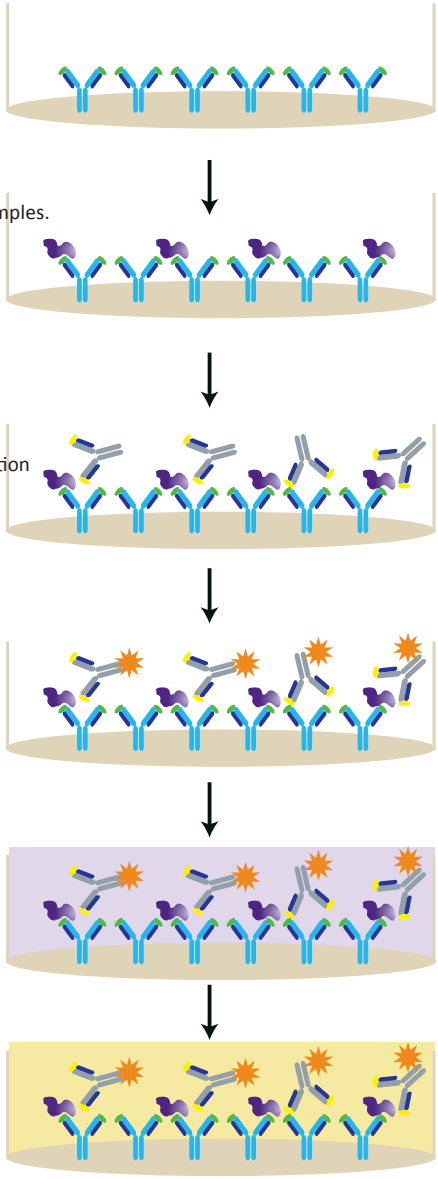
2. Add 50 µL prepared standards or samples.
Incubate 2 hrs, RT, shaking

3. Wash 4 times
Add 100 µL Detection Antibody solution
Incubate 1 hr, RT, shaking

4. Wash 4 times
Add 100 µL Avidin-HRP solution
Incubate 30 mins, RT, shaking

5. Wash 5 times
Add 100 µL Substrate Solution D
Incubate 15 mins, RT, in the dark

6. Add 100 µL Stop Solution



7. Read absorbance at 450 nm and 570 nm

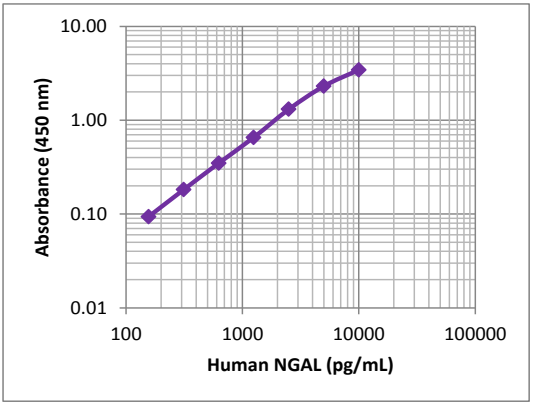
Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with antigen concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown antigen concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the sample concentration.

If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution as appropriate.

Typical Data:

This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Performance Characteristics:

Specificity: No cross-reactivity was observed when this kit was used to analyze the following recombinant cytokines/chemokines, each at 50 ng/mL.

Human	Lipocalin-1, MMP-9, MMP-1, MMP-3, TIMP-1, TIMP-2, PCSK9, IGF-1, TNF-α, TGF-β1, IL-4, IL-5, IL-11, IL-12(p70), IL-17A, IL-13, CCL2, CCL5, CCL14, CCL19
Mouse	NGAL

This kit is able to detect natural MMP-9/NGAL and natural MMP-9/TIMP-1/NGAL complexes in a linear fashion.

Sensitivity: The average minimum detectable concentration of human NGAL is 16.4 pg/mL.

Linearity: Ten serum, six plasma, five urine, and four saliva samples (all human) containing high concentrations of NGAL were diluted with Assay Buffer B to produce sample concentrations within the dynamic range of the assay. On average, 116%, 116%, 111% and 96% of the expected levels were detected from serum, plasma, urine, and saliva samples, respectively.

Recovery: Three levels of recombinant Human NGAL (2500 pg/mL, 625 pg/mL and 156 pg/mL) were spiked into ten human serum and five human urine samples, and analyzed with the LEGEND MAX™ Human NGAL ELISA Kit. On average, 91% and 80% of the protein were recovered from serum and urine samples, respectively.

Intra-Assay Precision: Two samples with different concentrations of human NGAL were tested with 16 replicates in one assay.

	Sample 1	Sample 2
Number of Replicates	16	16
Mean Concentration (pg/mL)	2454.0	234.1
Standard Deviation	182.6	13.7
% CV	7.4	5.8

Inter-Assay Precision: Two samples with different concentrations of human NGAL were assayed in four independent assays.

	Sample 1	Sample 2
Number of Assays	4	4
Mean Concentration (pg/mL)	2410.5	216.0
Standard Deviation	169.5	16.3
% CV	7.0	7.6

Biological Samples:

Serum, plasma, urine and saliva

Normal human serum, plasma, urine and saliva samples were tested for endogenous NGAL. The concentrations measured are shown below:

	Serum (n=23)	Heparin plasma (n=15)	EDTA plasma (n=13)	Citrate plasma (n=15)	Urine (n=8)	Saliva (n=10)
Detectable %	100	100	100	100	100	100
Mean (ng/mL)	202.2	206.3	259.0	173.0	7.2	2525.0
Maximum (ng/mL)	376.9	338.9	383.3	252.4	24.3	5929.0
Minimum (ng/mL)	102.5	47.8	100.4	105.4	1.8	915.0

Cell Culture Supernatant

The concentrations of various cell culture supernatant measured are shown in the table below.

Human peripheral blood mononuclear cells (PBMC) were incubated at 1 x 10^6 cells/mL in DMEM media with 10% fetal calf serum. Cells were unstimulated or stimulated with 10 µg/mL PHA and supernatants were removed five days after stimulation and assayed for human NGAL.

A431 human epithelial carcinoma cells were incubated in DMEM media with 10% fetal calf serum. The culture was unstimulated or stimulated with 1 µg/mL LPS. Supernatants were harvested one day after stimulation and assayed for human NGAL.

HT29 human colorectal adenocarcinoma cells were incubated in RPMI media with 10% fetal calf serum. Supernatants were removed on day 3 and assayed for human NGAL.

Sample	Treatment	Days	Concentration (pg/mL)
Control media	n/a	n/a	ND*
PBMC	Unstimulated	d5	39.4
	PHA stimulated		329.2
A431	Unstimulated	d1	11600.0
	LPS stimulated		14600.3
HT29	Unstimulated	d3	4232.1

*ND: Not detectable

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Troubleshooting Guide:

Problem	Probable Cause	Solution
High Background	Background wells were contaminated	Avoid cross-well contamination by using the provided plate sealers. Use multichannel pipettes and change tips between pipetting samples and reagents.
	Insufficient washes	Increase number of washes. Increase soaking time between washes prior to addition of substrate solution.
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
No or poor signal	Detection Antibody, Avidin-HRP or Substrate solution were NOT added	Rerun the assay and follow the protocol.
	Wrong reagent or reagents were added in wrong sequential order	
	Insufficient plate agitation	The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing.
	The wash buffer contains Sodium Azide (NaN3)	Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity.
	Incubations were done at an inappropriate temperature, timing or without agitation	Rerun the assay and follow the protocol.
Low or poor standard curve signal	The standard was incorrectly reconstituted or diluted	Adjust the calculations and follow the protocol.
	Standard was inappropriately stored	Store the reconstituted standard stock solution in polypropylene vials at -70°C. Avoid repeated freeze-thaw cycles.
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and the correct reagent volume.

Problem	Probable Cause	Solution
Signal is high, standard curves have saturated signal	Standard reconstituted with less volume than required	Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol.
	Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long	Rerun the assay and follow the protocol.
Sample readings are out of range	Samples contain no or below detectable levels of the analyte	If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications.
	Samples contain analyte concentrations greater than highest standard point	Samples may require dilution and analysis.
High variation in samples and/or standards	Multichannel pipette errors	Confirm that pipette calibrations are accurate.
	Plate washing was not adequate or uniform	Ensure pipette tips are tightly secured. Ensure uniformity in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before assaying.
	Samples may have high particulate matter	Remove particulate matter by centrifugation.
	Cross-well contamination	Do not reuse plate sealers. Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.

ELISA Plate Template

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

LEGEND MAX™ Kits are manufactured by **BioLegend Inc.**
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