NGAL (Lipocalin-2) ELISA

For the quantitative determination of NGAL in human stool and urine samples

For Research Use Only. Not For Use in Diagnostic Procedures.

Catalog Number: 43-NGALHU-E01
Size: 96 wells
Version: V8/US/2024-03 - ALPCO 1.0

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INTENDED USE

The NGAL ELISA is a quantitative immunoassay for the determination of human neutrophil gelatinase-associated lipocalin (NGAL or Lipocalin-2) in feces and urine. NGAL is extremely stable in feces. For Research Use Only. Not for Use in Diagnostic Procedures.

SUMMARY OF PHYSIOLOGY

NGAL or neutrophil gelatinase-associated lipocalin, also known as Lipocalin-2 (LCN2) or oncogene 24p3, is a protein encoded by the *LCN2* gene in humans.[1][2][3] NGAL is involved in innate immunity by sequestrating iron that in turn limits bacterial growth.[4] It is expressed in neutrophils and in low levels in the kidney, prostate, and epithelia of the respiratory and alimentary tracts.[3][5]

ASSAY PRINCIPLE

This ELISA kit is designed, developed, and produced for the quantitative measurement of human NGAL in stool samples. The assay utilizes the “sandwich” technique with selected antibodies that bind to various epitopes of NGAL.

Assay standards, controls, and samples are added directly to wells of a microtiter plate that is coated with antibody to human NGAL and incubated at room temperature for one hour. The plate is then washed and horseradish peroxidase (HRP) conjugated anti-NGAL is added to each well. After an additional incubation period, a “sandwich” of solid-phase polyclonal antibody - human NGAL – HRP conjugated antibody is formed. The unbound antibodies and buffer matrix are removed in the subsequent washing step. For the detection of this immunocomplex, the well is then incubated with a substrate solution in a timed reaction, which is terminated with an acidic reagent (i.e. ELISA stop solution). The absorbance is then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is directly proportional to the amount of human NGAL in the test sample. A standard curve is generated by plotting the absorbance versus the respective human NGAL concentration for each standard on a point-to-point or 4-parameter curve fit. The concentration of human NGAL in test samples is determined directly from this standard curve.

REAGENTS: Preparation and Storage

This test kit must be stored at 2-8°C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.

Anti-NGAL Antibody Coated Microplate – Ready to use

One microplate with twelve by eight strips (96 wells total) coated with polyclonal anti-human NGAL antibody. The plate is framed and sealed in a foil zipper bag with a desiccant. This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.

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ELISA Wash Concentrate
One bottle containing 30 mL of 30-fold concentrate. Before use the contents must be diluted with **870 mL** of demineralized water and mixed well. Upon dilution, this yields a working wash solution containing a surfactant in phosphate-buffered saline with a non-azide, non-mercury preservative. The 1X working wash solution may be stored at room temperature and is stable until the expiration date on the kit box.

ELISA HRP Substrate
One bottle containing 12 mL of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.

ELISA Stop Solution
One bottle containing 12 mL of stop solution. This reagent may be stored at 2 – 8°C or room temperature and is stable until the expiration date on the kit box.

Human NGAL Standards
Six vials containing recombinant human NGAL in a lyophilized bovine serum-based matrix with a non-azide preservative. **Refer to the vials for exact concentration of the standards.** These standards should be stored at 2-8°C and are stable until the expiration date on the kit box. Refer to assay procedure section for dilution directions.

Human NGAL Controls
Two vials containing human NGAL in a lyophilized bovine serum-based matrix with a non-azide preservative. **Refer to vials for exact concentration range for each control.** Both controls should be stored at 2-8°C and are stable until the expiration date on the kit box. Refer to assay procedure section for reconstitution instructions.

Tracer Antibody Diluent
One bottle containing **12 mL** ready-to-use buffer. It should be used only for tracer antibody dilution according to the assay procedure. This reagent should be stored at 2-8°C and is stable until the expiration date on the kit box.

HRP-Conjugated Anti-NGAL Antibody (Tracer Antibody)
One vial containing **0.6 mL** HRP-labeled anti-human NGAL antibody in a stabilized protein matrix. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box. **The tracer antibody should be prepared just prior to the end of the first incubation cycle.** Prepare 1X working Tracer Antibody by diluting NGAL Tracer Antibody 1:21 with the Tracer Antibody Diluent. Following is a table that outlines the relationship of strips used and antibody mixture to prepare.

Dilution Scheme	Tracer Antibody Diluent	Tracer Antibody
1	1 mL	50 µL
2	2 mL	100 µL
3	3 mL	150 µL

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4	4 mL	200 µL
5	5 mL	250 µL
6	6 mL	300 µL
7	7 mL	350 µL
8	8 mL	400 µL
9	9 mL	450 µL
10	10 mL	500 µL
11	11 mL	550 µL
12	12 mL	600 µL

Concentrated NGAL Fecal Extraction Buffer
One bottle containing **30 mL** of 20-fold concentrate. Before use the contents must be diluted with **570 mL** of demineralized water and mixed well. Upon dilution, this yields a 1X working Extraction Buffer for fecal and urine sample extraction and dilution. The 1X working Extraction Buffer may be stored at 2-8ºC and is stable for 2 months.

SAFETY PRECAUTIONS

The reagents must be used in a professional laboratory environment and are for research use only. The source material for reagents containing bovine serum albumin was derived in the contiguous 48 United States. It was obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they were potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Fecal sample collection tube (xxxxx)
2. Precision single channel pipettes capable of delivering 100 µL
3. Disposable pipette tips
4. Aluminum foil
5. Deionized or distilled water
6. Plastic microtiter well cover or polyethylene film
7. ELISA multichannel wash bottle or automatic (semi-automatic) washing system
8. Spectrophotometric microplate reader capable of reading absorbance at 450/650 or 450/620 nm

SAMPLE COLLECTION AND EXTRACTION

Only one fecal sample is required. Fresh fecal sample should be collected into a buffer-free container designed for stool collection. The collected fecal sample (raw stool) may be transported at ambient temperature, stored at room temperature or 2-8°C for 14 days. This

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fecal sample may be stored below -20 °C for a 1 year and is stable minimum with three freeze - thaw cycles. NGAL Stool Sample Collection Device Method:

The NGAL tube is specifically designed for easy collection of a substantially small amount of fecal sample into the tube pre-filled with sample extraction buffer. Follow the instructions for use with product 43-NGALEX-50. Following the standard extraction procedure, dilute each sample 1:10 with 1x NGAL Sample Extraction Buffer. For example, mixing 100 µl sample with 900 µl buffer in a clean test tube. This diluted sample can be used directly in the assay procedure.

The extracted stool sample should be tested within 8 hours or stored at 2-8°C and tested within 3 days. It may be stored below -20°C for a longer storage period. Avoid more than three freeze-thaw cycles.

Manual Stool Preparation:

The collected sample should be diluted in two steps at 1:40 and 1:90 with 1X working extraction buffer before measurement.

1. Label and tare an empty polypropylene tube together with an inoculation loop.
2. Weigh 50 – 100 mg of stool using the inoculation loop by placing it into the pre-tared tube.
3. Record the net amount of sample and break the inoculation loop; leave the lower part of the loop in the tube.
4. Add 1X working NGAL Extraction Buffer (39 parts of the stool volume, 1 g stool = 1 ml) into the tube:

Fecal Sample Weight (mg)	**Extraction Buffer Volume (ml)**
50	2.0
55	2.2
60	2.4
65	2.6
70	2.8
75	3.0
80	3.2
85	3.4
90	3.6
95	3.8
100	4.0

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5. Vortex to dissolve stool sample. Let the sample set at room temperature vertically for 30 min for sedimentation or centrifuge the sample at 3000 x g for 5 minutes.
6. Transfer 0.015 mL clear supernatant (no particles) to a clean tube containing 1.35 mL of 1X working NGAL Extraction Buffer. Mix the sample by gently vortexing. This extracted sample is ready to be measured for fecal NGAL.
7. The extracted stool sample should be tested within 8 hours or stored at 2-8°C and tested within 3 days. It may be stored below -20°C for a longer storage period. Avoid more than three freeze-thaw cycles.

Urine Sample Collection:

Only 50 µL of fresh urine sample is required for measurement of NGAL in duplicate. The collected sample must be stored at -20ºC if the assay is not to be performed within 24 hours. However, extracted urine samples can be stored at 2-8ºC and/or room temperature for up to 7 days. Avoid more than three freeze-thaw cycles.

Urine samples must be diluted before testing. Each urine sample should be diluted 1:20 with 1x working NGAL Extraction Buffer. For example, mix 50 µl sample with 950 µl 1X working buffer in a clean test tube. This diluted sample can be used directly in the assay procedure.

ASSAY PROCEDURE

Reagent Preparation

1. Prior to use, allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
2. Wash Concentrate and NGAL Extraction Buffer must be diluted to 1X working solution prior to use. Please see REAGENTS section for details.
3. Reconstitute all assay standards and controls by adding **1.0 mL** of demineralized water to each vial. Allow the standards and controls to sit undisturbed for 5 minutes, and then mix well by inversion and gentle vortexing. Make sure that all solids are dissolved completely prior to use. These reconstituted standards and controls must be stored at 2-8°C for up to three days or ≤ -20˚C for long-term storage. Do not exceed 3 freeze-thaw cycles.
4. Prepare Tracer Antibody just prior to the end of the first incubation cycle. Please see REAGENTS section for details.
5. Place a sufficient number of anti-NGAL antibody-coated microwell strips in a holder to run human NGAL standards, controls, and unknown samples in duplicate.

Assay Procedure

Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3	STRIP 4
A	STD 1	STD 5	SAMPLE 1	SAMPLE 5
B	STD 1	STD 5	SAMPLE 1	SAMPLE 5

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C	STD 2	STD 6	SAMPLE 2	SAMPLE 6
D	STD 2	STD 6	SAMPLE 2	SAMPLE 6
E	STD 3	C1	SAMPLE 3	SAMPLE 7
F	STD 3	C1	SAMPLE 3	SAMPLE 7
G	STD 4	C2	SAMPLE 4	SAMPLE 8
H	STD 4	C2	SAMPLE 4	SAMPLE 8

1. Add 100 µL of standards, controls, and diluted samples into the designated microwells.
2. Cover the plate with one plate sealer and also with aluminum foil and incubate the plate on a bench (no shaking) at room temperature for 1 hour ± 5 minutes.
   Note: Just prior to the end of the incubation time, dilute the proper amount of HRP-Conjugated Anti-NGAL Antibody (Tracer Antibody)
3. Remove aluminum foil and plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350 µL of 1x working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
4. Add 100 µL of the 1X working Tracer Antibody to each well.
5. Cover the plate with one plate sealer and also with aluminum foil and incubate the plate on a bench (no shaking) at room temperature for 30 minutes ± 5 minutes.
6. Remove aluminum foil and plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350 µL of 1x working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
7. Add 100 µL of HRP Substrate into each well.
8. Cover the plate with one plate sealer and also with aluminum foil and incubate the plate on a bench (no shaking) at room temperature for 20 minutes.
9. Immediately remove the aluminum foil and plate sealer and add 100 µL of Stop Solution into each well. Mix by gently tapping the edge of the plate.
10. Read the absorbance at 450 nm within a reference filter at 620 nm or 650 nm.

PROCEDURAL NOTES

1. It is recommended that all standards, controls, and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results.
2. Keep light-sensitive reagents in the original amber bottles.
3. Store any unused antibody-coated strips in the foil zipper bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate readings.
7. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
8. If adapting this assay to automated ELISA system such as DS-2 (Dynex Corp.), a procedural validation is necessary if there is any modification of the assay procedure.

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INTERPRETATION OF RESULTS

1. It is recommended to use a point-to-point or 4-parameter standard curve fit.
2. Calculate the average absorbance for each pair of duplicate test results.
3. Subtract the average absorbance of the level 1 standard (0 µg/g; 0 ng/mL) from the average absorbance of all other readings to obtain the corrected absorbance.
4. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer assisted data reduction programs may also be used for the calculation of results.
5. The human NGAL concentrations for the controls and fecal samples are read directly from the standard curve using their respective corrected absorbance. For human NGAL concentration for urine samples, values read directly from the urine standard curve should be multiplied by the dilution factor of 20.

   Note: NGAL ng/mL X 3.6 = NGAL µg/g stool
   NGAL µg/g stool X 0.278 = NGAL ng/mL

EXAMPLE DATA AND STANDARD CURVE FOR FECAL AND URINE SAMPLES

Typical absorbance data and the resulting standard curve from the NGAL ELISA analyzed for fecal samples are shown below. **This curve should not be used in lieu of a standard curve run with each assay.**

Well I.D.	Readings (OD 450)	Average (OD 450nm)	Corrected (-620nm)	Results
Std 1: 0 µg/g	0.017	0.017	0.000	
	0.017			
Std 2: 1.08 µg/g	0.106	0.104	0.087	
	0.102			
Std 3: 3.6 µg/g	0.256	0.254	0.237	
	0.252			
Std 4: 10.8 µg/g	0.679	0.687	0.670	
	0.694			
Std 5: 32.4 µg/g	1.597	1.605	1.588	
	1.614			
Std 6: 97.2 µg/g	2.810	2.846	2.829	
	2.881			
Control 1	0.491	0.468	0.451	7.2 µg/g
	0.446			
Control 2	1.245	1.251	1.234	24 µg/g
	1.257			

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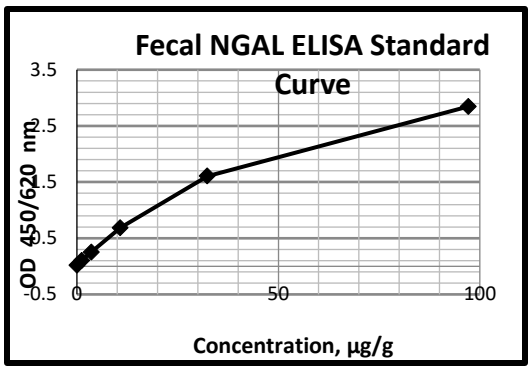
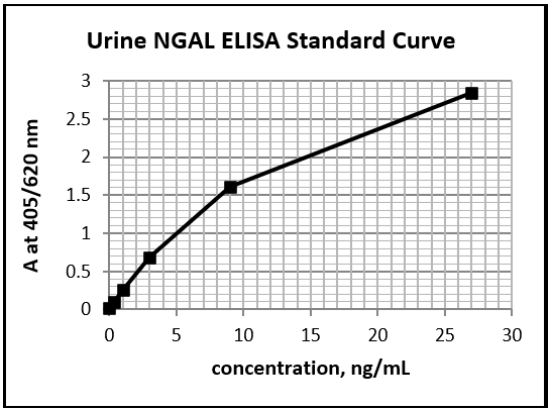


Typical absorbance data and the resulting standard curve from the NGAL ELISA analyzed for urine samples are shown below. **This curve should not be used in lieu of a standard curve run with each assay.**

Well I.D.	Readings (OD 450)	Average (OD 450nm)	Corrected (-620nm)	Results
Std 1: 0 ng/mL	0.017	0.017	0.000	
	0.017			
Std 2: 1.08 ng/mL	0.106	0.104	0.087	
	0.102			
Std 3: 3.6 ng/mL	0.256	0.254	0.237	
	0.252			
Std 4: 10.8 ng/mL	0.679	0.687	0.670	
	0.694			
Std 5: 32.4 ng/mL	1.597	1.605	1.588	
	1.614			
Std 6: 97.2 ng/mL	2.810	2.846	2.829	
	2.881			
Control 1	0.491	0.468	0.451	1.991 ng/mL
	0.446			
Control 2	1.245	1.251	1.234	6.688 ng/mL
	1.257			



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LIMITATION OF THE PROCEDURE

1. For sample values reading greater than the highest standard, it is recommended to re-assay samples with further dilution (1:10 or 1:100) with NGAL Sample Extraction Buffer.
2. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

QUALITY CONTROL

To assure the validity of the results, each assay should include adequate controls.

PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity of the human NGAL ELISA as determined by the 95% confidence limit on 16 duplicate determinations of zero standard is approximately 0.144 µg/g or 0.04 ng/mL.

High Dose “hook” effect

This assay has showed that it did not exhibit any high dose “hook” effect for NGAL levels up to 64,800 µg/g or 18,000 ng/mL.

Reproducibility and Precision

The intra-assay precision is validated by measuring three fecal control samples in a single assay with 16 replicate determinations:

Sample #	Mean NGAL Value (µg/g)	CV (%)
1	3.1	3.5
2	8.0	7.4
3	23.4	4.3

The inter-assay precision is validated by measuring two control samples in duplicate in 14 individual assays:

Sample #	Mean NGAL Value (µg/g)	CV (%)
1	7.3	5.3
2	22.0	4.6

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Linearity

Three human stool samples were collected, spiked with various amounts of NGAL, diluted with NGAL extraction buffer, and tested. The results of NGAL percent recovery in µg/g are as follows:

Dilution	Observed Value (µg/g)	% Recovery
Neat A	4.1	-
1:2	2.0	100.1
1:4	1.0	99.4
1:8	0.5	99.4
Neat B	2.7	-
1:2	1.3	95.2
1:4	0.7	102.7
1:8	0.3	100.0
Neat C	3.1	-
1:2	1.5	97.8
1:4	0.8	97.3
1:8	0.4	95.0

Two urine samples were collected, diluted with NGAL extraction buffer, and tested. The results of NGAL percent recovery in ng/mL are as follows:

Dilution	Observed Value (ng/mL)	% Recovery
Sample A 1:20	5.6	-
1:2	2.9	104
1:4	1.7	118
1:8	0.8	119
Sample B 1:20	0.5	-
1:2	0.2	93
1:4	0.1	92
1:8	0.05	85

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Spike Recovery
Three stool samples and four assay standards (1.2, 3.6, 10.8, and 32 µg/g) were combined at equal volumes and tested. The results are as follows:

Dilution	Observed Value (µg/g)	% Recovery
Neat A	0.14	-
Plus Std 2: 1.2 µg/g	0.6	87.8
Plus Std 3: 3.6 µg/g	1.7	88.6
Plus Std 4: 10.8 µg/g	5.1	93.3
Plus Std 5: 32.4 µg/g	15.8	97.1
Neat B	0.3	-
Plus Std 2: 1.2 µg/g	0.7	90.0
Plus Std 3: 3.6 µg/g	1.7	88.0
Plus Std 4: 10.8 µg/g	4.8	87.5
Plus Std 5: 32.4 µg/g	13.8	84.7
Neat C	0.2	-
Plus Std 2: 1.2 µg/g	0.6	80.7
Plus Std 3: 3.6 µg/g	1.7	88.0
Plus Std 4: 10.8 µg/g	4.6	83.0
Plus Std 5: 32.4 µg/g	14.2	87.0

Two diluted urine samples and three assay standards (1, 3, and 9 ng/mL) were combined at equal volumes and tested. The results are as follows:

Dilution	Observed Value (ng/mL)	% Recovery
Sample A 1:20	1.7	-
Plus Std 3: 1.0 ng/mL	1.3	92.3
Plus Std 4: 3.0 ng/mL	2.2	93.4
Plus Std 5: 9.0 ng/mL	5.2	96.4
Sample B 1:20	0.6	-
Plus Std 3: 1.0 ng/mL	0.7	95.7
Plus Std 4: 3.0 ng/mL	1.8	99.3
Plus Std 5: 9.0 ng/mL	4.5	94.8

WARRANTY

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. ALPCO DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall ALPCO be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser.

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