



EAGLE
BIOSCIENCES

NGAL (Stool) ELISA Assay Kit

Catalog Number:
NGL35-K01 (1 x 96 wells)
For Research Use Only. Not for use in diagnostic
procedures.

v. 7.1 (10 JUL .2023)

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INTENDED USE

The Eagle Biosciences Human NGAL ELISA Assay Kit is intended for use in the quantitative determination of human neutrophil gelatinase-associated lipocalin (Lipocalin-2 or NGAL) in feces. NGAL is extremely stable in feces. The Eagle Biosciences Human human neutrophil gelatinase-associated lipocalin (Lipocalin-2 or NGAL) ELISA Assay Kit is for research use only and not to be used in diagnostic procedures.

SUMMARY OF PHYSIOLOGY

NGAL or neutrophil gelatinase-associated lipocalin also known as Lipocalin-2 (LCN2) or oncogene 24p3 is a protein, which in humans is encoded by the LCN2 gene.^{[1][2][3]} NGAL is involved in innate immunity by sequestering 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]} Studies have shown that NGAL is an early biomarker for ischaemic renal injury after cardiopulmonary bypass.

ASSAY PRINCIPLE

This NGAL ELISA Assay 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 patient 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 fitting. The concentration of human NGAL in test samples is determined directly from this standard curve.

REAGENTS: Preparation and Storage

The NGAL ELISA Assay Kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit refer to the label on the NGAL ELISA Assay Kit box. All components are stable until this expiration date.



Prior to use allow all reagents to come to room temperature. Regents from different NGAL ELISA Assay Kit lot numbers should not be combined or interchanged.

1. Anti-NGAL Antibody Coated Microplate

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 NGAL ELISA Assay Kit box.

2. HRP Conjugated Anti-NGAL 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 NGAL ELISA Assay Kit box.

3. 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 nonazide, non-mercury preservative. The diluted wash solution may be stored at room temperature and is stable until the expiration date on the NGAL ELISA Assay Kit box.

4. 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 NGAL ELISA Assay Kit box.

5. 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 NGAL ELISA Assay Kit box.

6. 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 standard.** These standards should be stored at 2 – 8°C and are stable until the expiration date on the NGAL ELISA Assay Kit box. Refer to assay procedure section for dilution direction.

7. Human NGAL Controls

Two vials containing human NGAL in a lyophilized bovine serum based matrix with a nonazide 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 NGAL ELISA Assay Kit box. Refer to assay procedure section for reconstitution instructions.



8. 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 procedures. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the NGAL ELISA Assay Kit box.

9. Concentrated NGAL 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 ready-to-use Extraction Buffer for fecal sample extraction and dilution. The diluted Extraction Buffer may be stored at 2-8 °C and is stable for 2 months.

SAFETY PRECAUTIONS

The NGAL ELISA Assay reagents must be used in professional laboratory. Source material for reagents containing bovine serum 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 are 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. TMB is a suspected carcinogen. 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 (Eagle Biosciences Cat.# CAL35-C50 or similar)
2. Precision single channel pipettes capable of delivering 100 µL.
3. Disposable pipette tips suitable for above volume dispensing.
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.

SPECIMEN COLLECTION

Fecal Specimen Collection

1. Only one fecal sample is required. Fresh fecal sample must be collected by Fecal Sample Collection Tube (Eagle Biosciences Cat.# CAL35-C50 or similar). This tube is specially designed for easy collection of a substantially small amount of fecal sample into the tube pre-filled with sample extraction buffer. The collected fecal sample may be transported at ambient temperature, stored at room temperature or 2-8 °C for 14 days. This fecal sample may be stored below -20 °C for a 1 year and is stable minimum with three freeze - thaw cycles.

The validation data of this test were generated by using Fecal Sample Collection Tube (Eagle Biosciences Cat.# CAL35-C50). To order this tube please order Stool Sample Collection kit (Cat# CAL35-C50). Each kit contains 48 tubes filled with extraction butter. A different fecal NGAL test result may be obtained by using a different type of fecal sample collection tube.



2. It is an alternative to collect fecal sample with a commercial stool sample collection device. The collected sample can be stored at 2-8°C for up to 6 days. The collected sample should be diluted in two steps with 1:40 and 1:90 before measurement.

Following is a detailed sample extraction process.

- (a) Label and tare an empty polypropylene tube together with a inoculation loop.
- (b) Weigh 50 – 100 mg of stool using the inoculation loop by placing it into the pre-tared tube
- (c) Record the net amount of sample and break the inoculation loop; leave the lower part of the loop in the tube.
- (d) Add 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

- (e) 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.
- (f) Transfer 0.015 mL clear supernatant (no particles) to a clean tube with 1.35 ml Extraction Buffer. Mix the sample by gently vortexing. This extracted sample is ready to be measured for fecal Calprotectin.

Urine Sample Collection

1. Only 50 µL of fresh urine sample is required for measurement of NGAL measurement in duplicate. Urine specimen must be diluted before testing (see patient sample preparation for details). The collected sample must be stored at -20°C if the assay is not to be performed in 24 hours. However, extracted urine samples can be stored at 2-8°C and/or room temperature up to 7 days. Avoid more than three freeze-thaw cycles of specimen.



ASSAY PROCEDURE

1. Reagent Preparation

- (1) Prior to use allow all reagents to come to room temperature. Reagents from different NGAL ELISA Assay Kit lot numbers should not be combined or interchanged.
- (2) ELISA Wash Concentrate must be diluted to working solution prior to use. Please see REAGENTS section for details.
- (3) Reconstitute assay standards and controls by adding **1.0 mL** of demineralized water to each standard and control bottle. Allow the standard and controls to sit undisturbed for 5 minutes, and then mix well by inversions or gentle vortexing. Make sure that all solid is dissolved completely prior to use. These reconstituted standards and controls may be stored at 2- 8°C for up to 3 days or below -20 °C for long-term storage. Do not exceed 3 freeze-thaw cycles
- (4) Place a sufficient number of Anti-NGAL antibody-coated microwell strips in a holder to run human NGAL standards, controls and unknown samples in duplicates.
- (5) Prepare Tracer Antibody working solution by 1:21 fold dilution of the NGAL Tracer Antibody by adding the tracer antibody into the Tracer Antibody Diluent. Following is a table that outlines the relationship of strips used and antibody mixture prepared.

NOTE: the tracer antibody should be prepared just prior to the end of the first incubation cycle.

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

- (6) Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3	STRIP 4
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A	STD 1	STD 5	SAMPLE 1	SAMPLE 5
B	STD 1	STD 5	SAMPLE 1	SAMPLE 5
C	STD 2	STD 6	SAMPLE 2	SAMPLE 6
D	STD 2	STD 6	SAMPLE 2	SAMPLE 6
E	STD 3	C 1	SAMPLE 3	
F	STD 3	C 1	SAMPLE 3	
G	STD 4	C 2	SAMPLE 4	
H	STD 4	C 2	SAMPLE 4	

2. Patient Sample Preparation:

1. Patient samples collected with Fecal Sample Collection Tube (Cat. No. CAL35-K01). All patient samples should be diluted 1:10 with 1x Calprotectin/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.
2. Patient samples collected and extracted according to the specimen collection section #2. These samples don't require any further dilution and can be used directly in the assay procedure.

3. Assay Procedure:

- (1) Add **100 µl** of Standards, Controls and diluted patient samples (diluted beforehand with NGAL Sample Dilution Buffer) into the designated microwells.
- (2) Seal the plate wells securely, cover with foil or other material to protect from light. Incubate the plate static, at room temperature for **1 hr. ± 5 minutes**.
- (3) Just prior to the end of the incubation time, dilute the proper amount of Tracer Antibody for the assay.
- (4) Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
- (5) Add **100 µL** of the above Tracer Antibody to each well.
- (6) Seal the plate wells securely, cover with foil or other material to protect from light. Incubate the plate static, at room temperature for **30 minutes ± 5 minutes**.
- (7) Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
- (8) Add **100 µL** of ELISA HRP Substrate into each of the wells.



- (9) Cover the plate with aluminum foil or other material to avoid exposure to light. Incubate the plate static, at room temperature for **20 minutes**.
- (10) Immediately add **100 µL** of ELISA Stop Solution into each of the wells. Mix gently.
- (11) Read the absorbance at 450 nm with 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 reading.
7. All reagents of the NGAL ELISA Assay Kit should be mixed gently and thoroughly prior to use. Avoid foaming.
8. If adapting this assay to automated ELISA system such as DS-2 (Diamedix Corp., Miami), a procedural validation is necessary if there is any modification of the assay procedure.

INTERPRETATION OF RESULTS

It is recommended to use a point-to-point or 4-parameter standard curve fitting for the NGAL ELISA Assay Kit.

1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the level 1 standard (0 µg/g or 0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance.
3. 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.

The human NGAL concentrations for the controls and the patient fecal samples are read directly from the standard curve using their respective corrected absorbance.

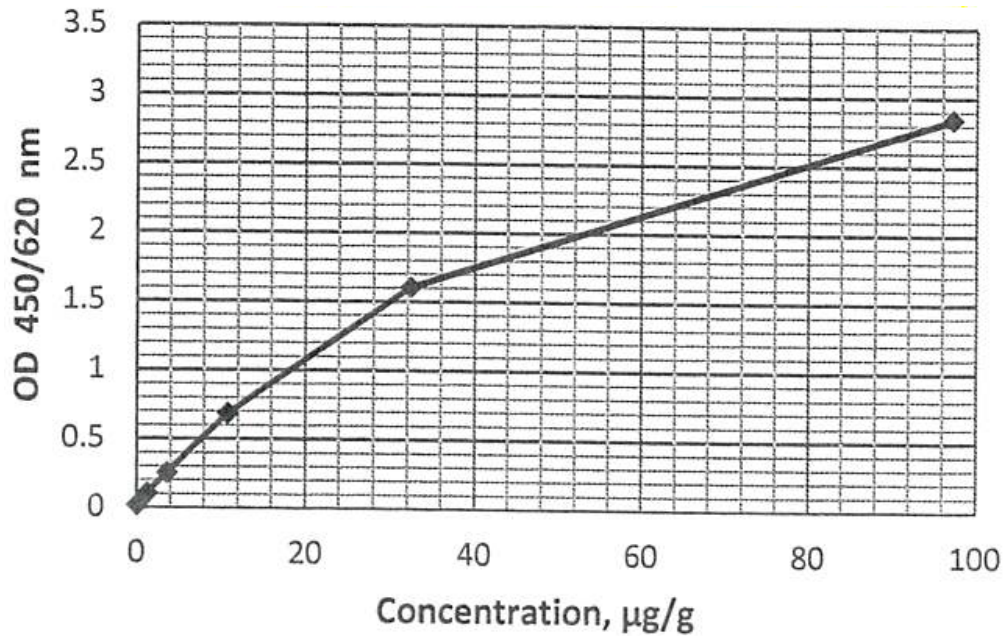
EXAMPLE DATA AND STANDARD CURVE

A typical absorbance data and the resulting standard curve from this Fecal NGAL ELISA are represented. **This curve should not be used in lieu of standard curve generated with each assay.**



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

Fecal NGAL ELISA Standard Curve





EXPECTED VALUES

Stool samples from normal healthy adults with age of 24 – 58 were collected and measured with this NGAL ELISA Assay Kit. The recommended normal cut-off for fecal NGAL concentration by using this ELISA and sample collection system is 25 µg/g directly read from assay standard curve. We strongly recommend that each clinical laboratory to establish its own normal cut-off level by measuring normal stool samples with this NGAL ELISA Assay Kit and sample collection system.

Please be aware that patients with recent diarrhea might give a much higher level of fecal NGAL. Taking spicy food or alcohol may also cause intestinal irritation resulting in an abnormal fecal NGAL level.

Note: $\text{NGAL ng/mL} \times 3.6 = \text{NGAL } \mu\text{g/g stool}$

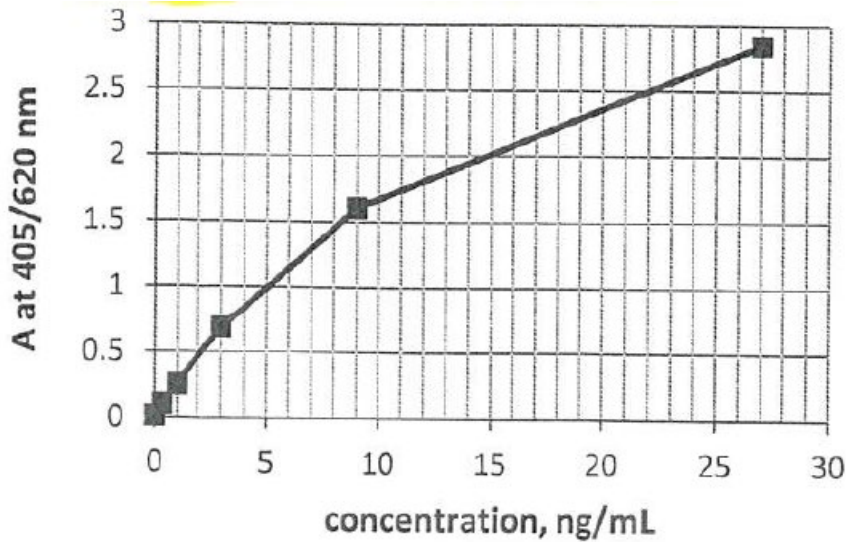
$\text{NGAL } \mu\text{g/g stool} \times 0.278 = \text{NGAL ng/mL}$

A typical absorbance data and the resulting standard curve from this Urine NGAL ELISA are represented. **This curve should not be used in lieu of standard curve generated with each assay.**

Well I.D.	OD 450/620 nm Absorbance			Results
	Readings	Average	Corrected	
Std-1: 0 ng/g	0.017 0.017	0.017	0.000	
Std-2: 0.3 ng/g	0.106 0.102	0.104	0.087	
Std-3: 1 ng/g	0.256 0.252	0.254	0.237	
Std-4: 3 ng/mL	0.679 0.694	0.687	0.670	
Std-5: 9 ng/mL	1.597 1.614	1.605	1.588	
Std-6: 27 ng/mL	2.810 2.881	2.846	2.829	
Control 1	0.491 0.446	0.468	0.451	1.991 ng/mL
Control 2	1.245 1.257	1.251	1.234	6.688ng/mL



Urine NGAL ELISA Standard Curve



EXPECTED VALUES

Urine samples from normal healthy adults with age of 20-58 were collected and measured with this ELISA. The recommended normal cut-off for urine NGAL concentration by using this ELISA is 50 ng/mL. (2.5 ng/mL read directly from assay standard curve multiplied by 20 (dilution factor)). We strongly recommend that each clinical laboratory to establish its own normal cut-off level by measuring normal urine samples with this ELISA.

LIMITATION OF THE PROCEDURE

1. An abnormally high NGAL test result cannot be independently used for clinical diagnosis. The same as other laboratory tests, a variety of analytical and pre-analytical factors may lead to false high test results. Physicians must interpret the test result in the light of each patient's clinical findings.
2. For sample values reading greater than the highest standard, it is recommended to reassay samples with further dilutions (i.e. 1:10 or 1:100 with NGAL Sample Extraction Buffer).
3. 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 (LLOD) of the NGAL ELISA Assay Kit as determined by the 95% confidence limit on 16 duplicate determination 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 have any high dose "hook" for NGAL levels up to 64,800 µg/g or 18,000 ng/mL.

Precision

The intra-assay precision was validated by measuring three control fecal samples 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 was validated by measuring two control levels in duplicate in 14 individual assays.

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

Linearity

Three **stool** samples were collected, spiked with various amounts of NGAL, diluted with Calprotectin/NGAL Extraction Buffer and tested with the NGAL ELISA Assay Kit. The results of NGAL percent recovery value in ng/mL are as follows:



DILUTION	OBSERVED VALUE ($\mu\text{g/mL}$)	% 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 NGAL extraction Buffer and tested. The results of NGAL percent recovery value 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%

Spike Recovery

Three stool samples and four assay standards (1.2, 3.6 and 10.8, 3.2 $\mu\text{g/g}$) were combined at equal volumes and tested. The results are as follows:



samples	OBSERVED VALUE $\mu\text{g/mL}$	% RECOVERY
Neat A	0.14	-
Std-2: 1.2 $\mu\text{g/ml}$	0.6	87.8%
Std-3: 3.6 $\mu\text{g/ml}$	1.7	88.6%
Std-4: 10.8 $\mu\text{g/ml}$	5.1	93.3%
Std-5: 32.4 $\mu\text{g/ml}$	15.8	97.1%
Neat B	0.3	-
Std-2: 1.2 $\mu\text{g/ml}$	0.7	90.0%
Std-3: 3.6 $\mu\text{g/ml}$	1.7	88.0%
Std-4: 10.8 $\mu\text{g/ml}$	4.8	87.5%
Std-5: 32.4 $\mu\text{g/ml}$	13.8	84.7%
Neat C	0.2	-
Std-2: 1.2 $\mu\text{g/ml}$	0.6	80.7%
Std-3: 3.6 $\mu\text{g/ml}$	1.7	88.0%
Std-4: 10.8 $\mu\text{g/ml}$	4.6	83.0%
Std-5: 32.4 $\mu\text{g/ml}$	14.2	87.0%

Two dilute urine samples and 3 assays standards (1, 3, and 9 ng/mL) were combined at equal volume and tested. The results are as follows:

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

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NGAL ELISA: Condensed Assay Protocol

1. 100 μ L Standards, controls and diluted patient samples
Incubate @ RT for 60 min static
Wash 5 x
2. 100 μ L Tracer Antibody
Incubate @ RT for 30 min static
Wash 5x
3. 100 μ L TMB Substrate
Incubate @ RT for 20 min static
4. 100 μ L Stop Solution
Immediately
5. Read absorbance at 450/650 or 450/620 nm
Within 10 minutes



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For further information about this kit, its application or the procedures in this kit, please contact the Technical Service Team at Eagle Biosciences, Inc. at info@eaglebio.com or at 866-411-8023.