

Human NGAL

ELISA Kit

KIT 036CE



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Please read these instructions carefully

INTENDED USE

For the in vitro determination of human NGAL in urine or plasma as a marker of acute kidney injury which may lead to acute renal failure.

INTRODUCTION

NGAL is a biomarker of kidney tubular damage and a biological response to the damage process which is distinctly separated from loss of kidney function called Acute Kidney Injury (AKI)¹. As damage often precedes loss of function renal expression of NGAL increases dramatically in a variety of causes and NGAL is released into both urine and plasma. Accordingly, renal disorders with presence of acute tubular damage are associated with raised urinary and plasma NGAL levels. Urinary NGAL serves as an early marker of acute kidney injury after cardiopulmonary bypass surgery^{2,3} and both urinary and plasma NGAL levels provide an early indication of acute renal injury in unselected patients in intensive care⁴. Raised urinary and plasma levels of NGAL have also been observed

in patients with chronic kidney diseases⁵, and persistently raised urinary levels after renal transplantation are associated with poor graft function⁶.

PRINCIPLE OF THE ASSAY PROCEDURE

The assay is a sandwich ELISA performed in microwells coated with a monoclonal antibody to human NGAL. Bound NGAL is detected with another monoclonal NGAL antibody labeled with biotin and the assay is developed with horseradish peroxidase (HRP)-conjugated streptavidin followed by the addition of a color-forming substrate. The assay is a 4-step procedure:

Step 1. Aliquots of calibrators, diluted samples and any controls are incubated in microwells pre-coated with monoclonal capture antibody. NGAL present in the solutions will bind to the coat, while unbound material is removed by washing.

Step 2. Biotinylated monoclonal detection antibody is added to each test well and incubated. The detection antibody attaches to bound NGAL; unbound detection antibody is removed by washing.

Step 3. HRP-conjugated streptavidin is added to each test well and allowed to form a complex with the bound biotinylated antibody. Unbound conjugate is removed by washing.

Step 4. A color-forming peroxidase substrate containing tetramethylbenzidine (TMB) is added to each test well. The bound HRP-streptavidin reacts with the substrate to generate a colored product. The enzymatic reaction is stopped chemically, and the color intensity is read at 450 nm in an ELISA reader. The color intensity (absorbance) is a function of the concentration of NGAL originally added to each well. The results for the calibrators are used to construct a calibration curve from which the concentrations of NGAL in the test specimens are read.

KIT COMPONENTS

Item	Contents	Quantity
1	12 x 8 coated Microwells + Frame	96 wells
2	5x Sample Diluent Conc.	50 mL
3	NGAL Calibrator 1-8. 0, 10, 25, 50, 100, 250, 500, 1000 pg/mL	8 x 1 mL
4	25x Wash Solution Conc.	1 x 40 mL
5	Biotinylated NGAL Antibody	1 x 12 mL
6	HRP-Streptavidin	1 x 12 mL
7	TMB Substrate	1 x 12 mL
8	Stop Solution	1 x 12 mL
9	NGAL Controls Low and High	2 x 50 μ L

Note: Liquid reagents contain preservatives and may be harmful if ingested.

MATERIALS REQUIRED BUT NOT PROVIDED

- Adjustable micropipettes covering the range 1-1000 μ L and corresponding disposable pipette tips
- Polypropylene tubes to contain up to 1000 μ L
- Tube racks
- Adjustable 8- or 12-channel micropipette (50-250 μ L range) or repeating micropipette (optional)
- Clean 1 L and 500 mL graduated cylinders
- Deionized or distilled water
- Cover for microwell plate
- Clean container for diluted Wash Solution
- Apparatus for filling wells during washing procedure (optional)
- Lint-free paper towels or absorbent paper
- Disposable pipetting reservoirs
- Timer (60-minute range)

- Calibrated ELISA plate reader capable of reading at 450 nm (preferably subtracting reference values at 650 or 620 nm)
- Sodium hypochlorite (household bleach 1:10 dilution) for decontamination of specimens, reagents, and materials

PRECAUTIONS**For *in vitro* diagnostic use only**

- This kit should only be used by qualified laboratory staff.
- Use separate pipette tips for each sample, calibrator and reagent to avoid cross-contamination.
- Use separate reservoirs for each reagent. This applies especially to the TMB Substrate.
- After use, decontaminate all specimens, reagents and materials by soaking for at least 30 minutes in sodium hypochlorite solution (household bleach diluted 1:10).
- To avoid droplet formation during washing, aspirate the wash solution into a bottle containing bleach.
- Avoid release into the environment. Dispose of containers and unused contents in a safe way and in accordance with national and local regulations.
- The Stop Solution contains 0.5 M sulfuric acid and can cause irritation or burns to the skin and eyes. If contact occurs, rinse immediately with plenty of water and seek medical advice.
- Do not interchange components from kits with different batch numbers. The components have been standardized as a unit for a given batch.
- Hemolyzed, hyperlipemic, heat-treated or contaminated specimens may give erroneous results.
- Do not dilute specimens directly in the microwells.
- Do not touch or scrape the bottom of the microwells when pipetting or aspirating fluid.
- Incubation times and temperatures other than those specified may give erroneous results.

13. Do not allow the wells to dry once the assay has begun.
14. The TMB Substrate is light sensitive. Keep away from bright light.
15. Do not reuse microwells or pour reagents back into their bottles once dispensed.

STABILITY AND STORAGE

1. Store the kit with all reagents at 2-8°C. Do not freeze.
2. Use all unopened reagents before the expiry date on the kit box label.
3. The stability of materials/reagents after opening is indicated on the kit box label.
4. Diluted Wash Solution Concentrate remains stable for 4 weeks at 2-8°C. If not using all wells, dilute only the portion of Wash Solution Concentrate required.
5. Diluted Sample Diluent Concentrate remains stable for 4 weeks at 2-8°C. If not using all wells, dilute only the portion of Sample Diluent Concentrate required.
6. For subsequent use, store unused wells in the foil pouch with the desiccant provided and reseal. Always allow the foil pouch to equilibrate to room temperature before opening to avoid condensation in/on the coated microwells.

COLLECTION OF SPECIMENS

Handle and dispose of all blood-derived or urine specimens as if they were potentially infectious. See Precautions, sections 1, 2, 4 and 5.

Determination of NGAL in a single specimen requires 10 µL of fluid sample. Blood specimens should be collected aseptically into heparinized or EDTA tubes by qualified staff using approved venipuncture techniques. Plasma should be prepared by standard techniques for laboratory testing. Urine should be centrifuged. Samples can be kept cool at 2 - 8°C for two days. For storage of specimens, -70°C or below is recommended. Do not use hemolyzed, hyperlipemic, heat-treated or contaminated specimens.

PREPARATION OF REAGENTS AND SAMPLES

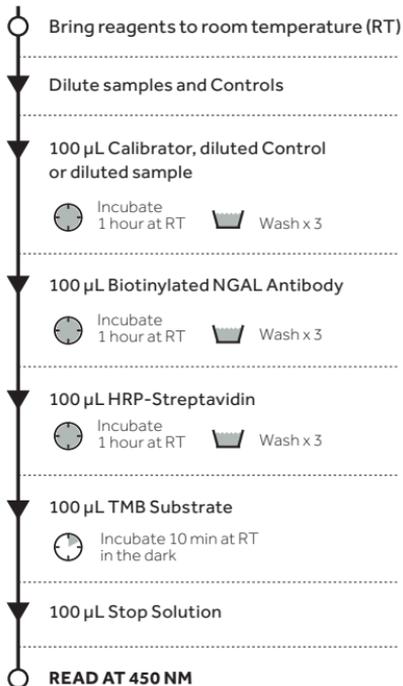
1. Bring all specimens and reagents to room temperature (20-25°C). Mix specimens thoroughly by gentle inversion and if necessary clear visible particulate matter by low-speed centrifugation.
2. Determine the number of specimens to be tested (in duplicate), NGAL control Low and High (in duplicate), any internal laboratory control specimens (in duplicate) plus any reagent blank wells. The pre-coated wells can be used as strips of 8 or as individual wells. Single wells are handled by breaking individual wells apart and placing each well in the frame at an appropriate position. Letters and notches on the wells allow the individual wells to be identified. Add 16 wells for the 8 calibrators (in duplicate). Remove the number of microwells required and replace the remainder in the foil pouch with desiccant at 2-8°C.
3. Wash Solution: Dilute the 25x Wash Solution Concentrate by pouring the total contents of the bottle (40 mL) into a 1-L graduated cylinder and add distilled or deionized water to a final volume of 1 L. Mix thoroughly and store at 2-8°C. If not all the wells are to be used, dilute (1/25) only the volume of Wash Solution Concentrate required.
4. Sample Diluent: Dilute the 5x Sample Diluent Concentrate (contains red dye to aid pipetting) by pouring the total contents of the bottle (50 mL) into a 250-mL graduated cylinder and add distilled or deionized water to a final volume of 250 mL. Mix thoroughly and store at 2-8°C. If not all the wells are to be used, dilute (1/5) only the volume of Sample Diluent Concentrate required.
5. NGAL Calibrators (contains red dye to aid pipetting): The assigned concentrations are indicated on their labels. Do not dilute further.
6. Biotinylated NGAL Antibody (ready to use): Do not dilute further.
7. HRP-Streptavidin Conjugate (ready to use): Do not dilute further.
8. TMB Substrate (ready to use): Do not dilute further.

9. Stop Solution (ready to use): Do not dilute further.
10. NGAL Controls Low and High: Dilute each control with the pre-diluted Sample Diluent to obtain at least 250 μL of diluted solution that can be set up in duplicate wells at 100 μL per well. Dilute the controls 1/500. This can be prepared in two steps, as follows: dilute 10 μL of sample in 190 μL of Sample Diluent to make a 1/20 dilution; then dilute 10 μL of the 1/20 dilution in 240 μL of Sample Diluent to make a 1/500 dilution. The target values of Control Low and Control High can be found on the vial label.
11. Specimens: Dilute each specimen in a recorded proportion with the pre-diluted Sample Diluent to obtain at least 250 μL of diluted solution that can be set up in duplicate wells at 100 μL per well. An initial screening at a dilution of 1/500 is recommended for physiological fluids. This can be prepared in two steps, as follows: dilute 10 μL of sample in 190 μL of Sample Diluent to make a 1/20 dilution; then dilute 10 μL of the 1/20 dilution in 240 μL of Sample Diluent to make a 1/500 dilution. Dilutions are mixed by inversion or moderate vortexing. Re-assay of out-of-range samples at lower or higher dilution is rarely necessary. Dilutions lower than 1/10 should not be used.

ASSAY PROCEDURE

1. Prepare the assay protocol, assigning the appropriate wells for setting up calibrators, diluted NGAL controls, diluted specimens and any internal laboratory controls in duplicate. If a reference wavelength of 650 or 620 nm is not available on the ELISA reader, a reagent blank well can be assigned. This is set up with 100 μL of Sample Diluent instead of diluted specimen and processed like the other wells.
2. Pipette 100 μL volumes of each calibrator, diluted NGAL controls, diluted specimens and any internal laboratory controls into the corresponding positions in the microwells. Cover the wells and incubate for 60 minutes at room temperature on a shaking platform set at 200/minute.
3. Aspirate the contents of the microwells and wash the microwells three times with 300 μL diluted Wash Solution. If washing is done manually, empty the microwells by inversion and gentle shaking into a suitable container, followed by blotting in the inverted position on a paper towel. A dwell time of 1 minute before emptying is recommended for at least the last wash of the cycle.
4. Dispense 100 μL of Biotinylated NGAL Antibody (ready to use) into each microwell. A multichannel or repeating micropipette can be used. Cover the wells and incubate for 60 minutes at room temperature on a shaking platform (200/minute).
5. Wash as described above in Step 3.
6. Dispense 100 μL of HRP-Streptavidin Conjugate (ready to use) into each microwell. A multichannel or repeating micropipette can be used. Cover the wells and incubate for 60 minutes at room temperature on a shaking platform (200/minute).
7. Wash as described above in Step 3.
8. Dispense 100 μL of TMB Substrate (ready to use) into each microwell. The use of a multichannel micropipette is recommended to reduce pipetting time. Cover the wells and incubate for **exactly 10 minutes** at room temperature in the dark. Start the clock when filling the first well.
9. Add 100 μL Stop Solution (ready to use) to each well, maintaining the same pipetting sequence and rate as in Step 8. Mix by gentle shaking for 20 seconds, avoiding splashing. Read the wells within 30 minutes.
10. Read the absorbances of the wells at 450 nm in an appropriate microplate reader (reference wave-length 650 or 620 nm). If no reference wavelength is available, the value of the reagent blank well is subtracted from each of the other values before other calculations are performed.

SCHEMATIC OVERVIEW



CALCULATION OF RESULTS

A calibration curve is constructed by plotting the absorbance values obtained for the calibrators on the y-axis against the corresponding NGAL concentrations the x-axis. The calibration curve must meet the validation requirements. The NGAL concentrations of diluted samples are then found by placing their absorbance values on the calibration curve and reading the corresponding concentrations from the x-axis.

This procedure can be performed manually using graph paper with linear x and y scales. A smooth curve can be drawn through the points or adjacent points can be joined by straight lines. The latter procedure may slightly overestimate/underestimate concentration values between points when the curve is slightly convex to left/right, respectively. Although the curve may approximate to a straight line, it is both practically and theoretically incorrect to calculate and draw the straight line of best fit and to read the results from this.

The procedure can also be performed by an ELISA reader software program incorporating curve fitting procedures. The procedure of choice is to use linear x and y axes with 4-parameter logistic curve fitting. Diluted samples that give a mean absorbance above that for the 1000 pg/mL NGAL Calibrator or below that for the 10 pg/mL NGAL Calibrator are out of the range of the assay and their concentrations should be noted as >1000 pg/mL and <10 pg/mL respectively. The corresponding concentrations in the undiluted samples are calculated and reported as >(1000 x dilution factor) pg/mL and <(10 x dilution factor) pg/mL, respectively. If necessary, these samples can be re-assayed at higher and lower dilutions for high- and low-reading samples, respectively. The new dilution factors should be those estimated to give absorbance values that fall well within the range of the calibration curve, but dilutions lower than 1/10 should not be used.

VALIDATION OF CALIBRATION CURVE

The mean absorbance for the 1000 pg/mL NGAL Calibrator should be >1.5 . The mean absorbance for any NGAL calibrator should be higher than that for the previous NGAL calibrator, e.g. absorbance(100 pg/mL NGAL) $>$ absorbance(50 pg/mL NGAL). The curve should be slightly convex to the left when the results are plotted on linear axes.

CALIBRATION TROUBLESHOOTING

Out-of-line points for individual calibrators: One or more individual calibrators may give anomalous absorbance readings. One or both of the duplicate values may be out of line, and the mean of the duplicates may be out of line. This error is significant if it impairs satisfactory curve fitting by the 4-parameter logistic method, which, as a result of the anomalous value, is shifted away from other calibrator points that are in fact correct. The calibrator points and fitted curve should always be examined for correct fit before any calculations of concentration from it are accepted. A poorly fitting curve will also be revealed by a high sum of residual squares. If only one calibrator is affected, which is not the highest calibrator, two courses of action are possible:

- i) An erroneous singlet or duplicate result should be eliminated from the curve, and the remaining results refitted by the 4-parameter logistic procedure. If a satisfactory fit is obtained, provisional concentration results can be calculated from it.
- ii) If no satisfactory fit can be obtained in this way, but the curve is otherwise consistent, provisional results can be obtained from straight lines or simple cubic spline fitting between the means of duplicates, omitting the erroneous point.

If two or more calibrators are affected, the assay should be repeated.

A deviant result for an individual calibrator can be due to operator error or to calibrator deterioration. If both duplicate values are consistently out of line in successive assays, the calibrator is faulty and should be omitted.

TRACEABILITY OF CALIBRATOR VALUE

No internationally approved reference material for NGAL is currently available. The NGAL concentration of calibrator material has been assigned by turbidimetry using a precise transfer protocol ensuring traceability to the BioPorto Diagnostics master calibrator. The reference material was value-assigned by measurement of light absorbance at 280 nm using a theoretically calculated extinction coefficient based on the amino-acid composition.

INTERPRETATION OF RESULTS

Urinary and/or plasma concentrations of NGAL may be elevated in conditions that show no apparent relation to change in kidney function, including bacterial infections, other inflammatory disorders and certain carcinomas. BioPorto Diagnostics has determined that the NGAL concentration in an isolated sample of urine and/or plasma should exceed 250 ng/mL in order to indicate the presence of renal disorder, including acute kidney injury, without incurring the risk of an unacceptably high proportion of false positive diagnoses of renal disorder.

QUALITY CONTROL

For quality control, use the Controls included in the kit. Quality control intervals and limits should be adapted to each laboratory's individual requirements. Each laboratory should establish corrective measures if values fall outside the limits.

LIMITATIONS

The finding of a raised urinary or plasma level of NGAL cannot be independently diagnostic of any single pathology. A variety of independent pathologies are associated with raised levels of urinary or plasma NGAL. Physicians must interpret the significance of any raised NGAL level in the light of each patient's clinical features.

NORMAL REFERENCE RANGE

The NGAL level was measured in samples from equal amounts of male and female self-declared healthy individuals. Ages ranging from 22 to 67 years, median 41.

Matrix	Median NGAL (ng/mL)	95% confidence Interval (ng/mL)	Sample number
Urine	25.3	2.4–154	120
Li-Heparin	60.9	39.5–97.5	120
K3-EDTA	62.1	40.8–104	40

In unselected patients admitted to intensive care, the NGAL concentrations ranged from 9 ng/mL to 40,000 ng/mL (40 µg/mL) in urine (n = 60) and from 25 ng/mL to 3490 ng/mL in EDTA plasma (n = 60).

PERFORMANCE CHARACTERISTICS

Measuring range: The measuring range of the kit is 10-1000 pg/mL

Limit of detection: The lowest concentration of NGAL giving an absorbance reading greater than 2 SD above the mean zero (NGAL Calibrator 1) reading (n = 20) was determined to be 1.4 pg/mL, this being lower than the value of NGAL Calibrator 2.

Precision: Intra-assay variation was determined by measurement of NGAL in two urine samples and two plasma samples with 6-8 replicates. The following results were obtained (CV = coefficient of variation):

Sample	CV
Urine A	14%
Urine B	7%
Plasma A	11%
Plasma B	10%

Inter-assay variation was determined by measurement of NGAL in 2 diluted urine samples and 2 diluted EDTA plasma samples with 2-8 replicates in 2-4 separate assays. The following results were obtained:

Sample	CV
Urine A	4%
Urine B	8%
Plasma A	4%
Plasma B	14%

Analytical recovery: Urine and plasma samples were spiked with recombinant human NGAL and analyzed in the assay. Recovery was calculated from (Measured/Expected) expressed as a percentage.

Sample	Measured (range)	Expected (range)	Recovery (range)
Urine	160 - 494 pg/mL	174 - 618 pg/mL	80%-100%
Plasma	159 - 532 pg/mL	177 - 621 ng/mL	86%-99%

Linearity: NGAL was measured in serial dilutions (n = 7-8) of 2 urine samples and 2 plasma samples. The CV of the mean of the measured values corrected for the dilution was 7% and 11% for the two urine samples and 13% and 14% for the two plasma samples, demonstrating satisfactory linearity.

Sample material: Analysis of samples of urine or EDTA plasma showed no significant differences in analytical recovery, linearity or precision. However, serum samples are not recommended for the assessment of acute kidney injury as NGAL is released from neutrophils during blood clotting and may augment values irrespective of kidney injury⁷.

Specificity: The two monoclonal antibodies against human NGAL used in the assay have been shown to bind to different preparations of recombinant human NGAL and to give a single band at 25 kDa on Western blot analysis of a reduced post-nuclear supernatant from human neutrophils⁸.

LIABILITY

This ELISA Kit is only intended for the in vitro determination of NGAL in human urine or plasma. The ELISA Kit is only intended for use by qualified personnel carrying out research or diagnostic activities. If the recipient of this test passes it on in any way to a third party, this instruction must be enclosed, and said recipient shall at recipient's own risk secure in favor of BioPorto Diagnostics A/S all limitations of liability herein.

REFERENCES**LITERATUR****RÉFÉRENCES****BIBLIOGRAFIA****BIBLIOGRAFIA****REFERENCER****REFERENSER**

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This product is protected by one or more U.S., European and/or foreign patents as listed on <http://www.bioporto.com/about-us/patents>.

	Catalogue number		Concentrated Sample Diluent. Dilute before use.
	In vitro diagnostic medical device		Concentrated Wash Solution. Dilute before use.
	Batch code		Control Low
	Valid version of instructions		Control High
	Consult instructions for use		
	European Conformity		
	Use by		
	Once opened, use within the specified number of months		
	Manufacturer		
	Keep away from sunlight		
	Temperature limitation		
	Do not reuse		
	Caution, consult accompanying documents		
	Biological risk		
	Do not use if package is damaged		

RELATED PRODUCTS

Cat. No.	Product name
KIT 042	Mouse NGAL ELISA Kit
KIT 043	Dog NGAL ELISA Kit
KIT 044	Pig NGAL ELISA Kit
KIT 045	Monkey NGAL ELISA Kit
KIT 046	Rat NGAL ELISA Kit
KIT 048	Human NGAL monomer-specific ELISA Kit



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