NGAL ELISA Kit

EN

KIT 036

BioPorto® Diagnostics

Please read these instructions carefully

INTENDED USE

For the *in vitro* determination of human NGAL in tissue fluids (e.g. plasma, serum or urine), tissue extracts or culture media. For research use only.

INTRODUCTION

NGAL[1] (neutrophil gelatinase-associated lipocalin) belongs to the lipocalin family of proteins. These are typically small secreted proteins characterized by their ability to bind small, hydrophobic molecules in a structurally conserved pocket formed by β-pleated sheet, to bind to specific cell-surface receptors and to form macromolecular complexes. NGAL has many synonyms: it also known as NL (neutrophil lipocalin; HNL: human NL),[2] lipocalin 2, oncogene protein 24p3[3] or uterocalin[4] (in the mouse) and neu-related lipocalin[5] or 25 kDa α_2-microglobulin-related protein[6] (in the rat). Human NGAL consists of a single disulfide-bridged polypeptide chain of 178 amino-acid residues with a calculated molecular mass of 22 kDa,[1] but glycosylation increases its apparent molecular mass to 25 kDa. In neutrophils (neutrophilic polymorpho-nuclear leukocytes) and urine it occurs as monomer, with a small percentage of dimer and trimer, and also in complex with 92-kDa human neutrophil type IV collagenase, also called gelatinase B or matrix metalloproteinase-9 (MMP-9).[7]

NGAL was originally isolated from the supernatant of activated human neutrophils,[1] but it is also expressed at a low level in other human tissues including the kidney, prostate and epithelia of the respiratory and alimentary tracts.[8,9] It is strongly expressed in adenomas and inflamed epithelia of the bowel,[10] adenocarcinomas of the breast,[11] and urothelial carcinomas.[12]

Because of its small molecular size and resistance to degradation, NGAL is readily excreted and detected in the urine, both in its free form and in complex with MMP-9. Urinary levels correlate with plasma or serum levels whatever the cause of increased NGAL production (BioPorto Diagnostics data), but particularly high urinary levels can be expected when it is released directly into the urine by the kidney tubules or urothelial carcinomas. It is uncertain how far NGAL-MMP-9 complexes from sources remote from the urinary tract are excreted as such into the urine or reform in the urine after independent excretion of NGAL and MMP-9.[7]

While the functions of NGAL are not fully understood, it appears to be upregulated in cells under “stress”, e.g. from infection, inflammation, ischemia or neoplastic transformation, or in tissues undergoing involution, such as the postpartum mouse uterus and mammary glands on weaning. In relation to a possible antibacterial role, it binds enterobactin and other siderophores, depriving the microorganisms of Fe^{3+}, an important nutritional requirement.[13] Its complex formation with MMP-9 appears to protect MMP-9 enzymatic activity from degradation.[7] The upregulation of NGAL in involuting tissues has led to the postulation of a role in apoptosis, but it appears more likely that NGAL is associated with a survival response.[14] This seems to be so in the kidney, where NGAL-siderophore-iron complex rescues the mouse kidney from ischemic injury.[15]

NGAL in inflammation/infection. NGAL is released from the secondary granules of activated neutrophils[1] and plasma levels rise in inflammatory or infective conditions, especially in bacterial infections.[16] Thus the level of NGAL in plasma or serum has been proposed as a marker of infection. However, as levels of NGAL may also be raised in neoplastic conditions and renal disorders independently of any infective process, this proposed application should be treated with caution. NGAL may also be raised in infections in patients with an uncountably low number of neutrophils due to leukemia or treated leukemia, showing that the source of the raised NGAL in infections is not only the neutrophils. Indeed, serum NGAL levels correlate very poorly with the neutrophil count in patients with varying degrees of infection or inflammation (BioPorto Diagnostics data).

NGAL and neoplasia. The various types of cancer in which NGAL may be upregulated (often with MMP-9) have been referred to above. This has been shown by its expression in tumor cells and its high urinary levels, both in the free form and complexed with MMP-9.[7] Indeed, it has been proposed that

urinary NGAL-MMP-9 complexes may serve as a marker of disease status for breast cancer patients.[17] Plasma levels have not usually been measured in these cases.

NGAL and the kidney. Even before NGAL had been isolated from human neutrophils, its mouse homologue 24p3 was known to be expressed by kidney cells and to undergo an early, dramatic upregulation (14- to 20-fold) in response to SV 40 viral infection.[18] A similar early and dramatic upregulation was later observed in rat proximal tubule cells after ischemia-reperfusion injury,[19] and raised plasma levels of NGAL were found to be strongly correlated with decreased renal function in patients with renal damage due to systemic vasculitis.[20] The results for renal ischemia-reperfusion injury were subsequently confirmed and extended to nephrotoxic agents.[21,22,23] It has been suggested that urinary NGAL levels may serve as an early marker for ischemic renal injury in children after cardiopulmonary bypass.[24] Raised urinary and serum NGAL levels have also been observed in patients with established renal failure (BioPorto Diagnostics data) and patients with functioning renal grafts also showed raised urinary levels (detectable by Western blotting).[12] It is therefore apparent that a large variety of renal disorders are associated with raised plasma and urinary levels of NGAL. While plasma and urinary NGAL levels are closely correlated in acute conditions, it is to be expected that urinary NGAL levels will be particularly high after ischemic renal injury severe enough to result in acute renal failure, acute tubular necrosis or acute tubulo-interstitial nephropathy. However, the use of urinary NGAL as a potential marker for these conditions is subject to the proviso that other concurrent conditions that are independently associated with raised NGAL levels are taken into account.

NGAL as a potential diagnostic marker. The finding of a raised urinary or plasma level of NGAL cannot be independently diagnostic of any single pathology. As stated above, a variety of independent pathologies are associated with raised levels of urinary or plasma NGAL. For this reason the present kit is presented for research use only.

PRINCIPLE OF THE ASSAY

The assay is a sandwich ELISA performed in microwells coated with a monoclonal antibody against human NGAL. Bound NGAL is detected with another monoclonal antibody labeled with biotin and the assay is developed with horseradish peroxidase (HRP)-conjugated streptavidin and 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 precoated 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	12x 8 coated Microwells (96 wells) + frame	1 plate
②	5x Sample Diluent Conc.	60 mL
③	NGAL Calibrators (ready to use). 0, 10, 25, 50, 100, 250, 500, 1000 pg/mL	8 x 1 mL
④	25x Wash Solution Conc.	1 x 30 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 16 mL

Note: Liquid reagents contain the preservatives sodium azide, thimerosal or Bronidox L. These may be harmfull if ingested.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Adjustable micropipettes covering the range 1-1000 µL and corresponding disposable pipette tips
2. Polypropylene tubes to contain up to 1000 µL
3. Tube racks
4. Adjustable 8- or 12-channel micropipette (50-250 µL range) or repeating micropipette (optional).
5. Clean 1 L and 500 mL graduated cylinders
6. Deionized or distilled water
7. Cover for microplate
8. Clean container for diluted Wash Solution
9. Apparatus for filling wells during washing procedure (optional)
10. Lint-free paper towels or absorbent paper
11. Disposable pipetting reservoirs
12. Timer (60-minute range)
13. Calibrated ELISA plate reader capable of reading at 450 nm (preferably subtracting reference values at 650 or 620 nm)
14. Sodium hypochlorite (household bleach 1:10 dilution) for decontamination of specimens, reagents, and materials

PRECAUTIONS

For *in vitro* research use only

1. This kit should only be used by qualified laboratory staff.
2. Use separate pipette tips for each sample, calibrator and reagent to avoid cross-contamination.
3. Use separate reservoirs for each reagent. This applies especially to the TMB Substrate.
4. After use, decontaminate all specimens, reagents and materials by soaking for at least 30 minutes in sodium hypochlorite solution (household bleach diluted 1:10).
5. To avoid droplet formation during washing, aspirate the wash solution into a bottle containing bleach.
6. Avoid release into the environment. Dispose of containers and unused contents in a safe way and in accordance with national and local regulations.
7. The 5x Sample Diluent Concentrate is preserved with 0.25% sodium azide (corresponding to 0.05% in final diluted solution). Sodium azide is harmful in contact with skin and if swallowed. Sodium azide is harmful to aquatic organisms and may cause long-term adverse effects in the aquatic environment. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). According to EU regulations, no danger labeling is necessary for the diluted solution.
8. 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.
9. Do not interchange components from kits with different batch numbers. The components have been standardized as a unit for a given batch.
10. Hemolyzed, hyperlipemic, heat-treated or contaminated specimens may give erroneous results.
11. Do not dilute clinical specimens directly in the microwells.
12. Do not touch or scrape the bottom of the microwells when pipetting or aspirating fluid.
13. Incubation times and temperatures other than those specified may give erroneous results.

14. Do not allow the wells to dry once the assay has begun.
15. The TMB Substrate is light sensitive. Keep away from bright light.
16. 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 reagents before the expiry date on the vial labels.
3. 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.
4. Diluted Sample Diluent Concentrate remains stable for 5 days at 2-8°C. If not using all wells, dilute only the portion of Sample Diluent Concentrate required.
5. For subsequent use, store unused wells in the foil pouch with the desiccant provided and reseal. Always allow 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 serum, plasma or urine. Blood specimens should be collected aseptically into plain, heparinized or EDTA tubes by qualified staff using approved venepuncture techniques. Serum or plasma should be prepared by standard techniques for clinical laboratory testing. Urine should be centrifuged. Cap the prepared clinical specimens and freeze them at –20°C or below. This especially applies if the assay cannot be performed within 24 hours or if the specimen is to be shipped. For long-term storage of clinical specimens, –70°C or below is recommended. **Avoid repeated freezing and thawing.** Do not use hemolyzed, hyperlipemic, heat-treated or contaminated specimens.

PREPARATION OF REAGENTS AND SAMPLES

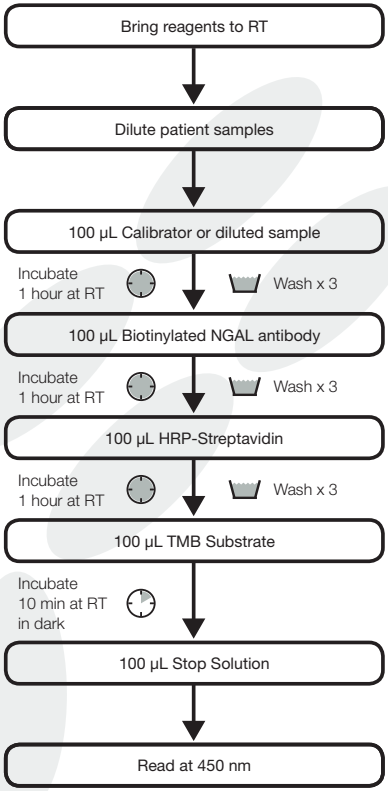
1. Bring all specimens and reagents to room temperature (20-25°C). Mix specimens thoroughly by gentle inversion and if necessary clear visible particulate matter by low-speed centrifugation.
2. Determine the number of specimens to be tested (in duplicate) plus any internal laboratory control specimens (in duplicate) plus any reagent blank wells. The precoated 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 (30 mL) into a 1-L graduated cylinder and add distilled or deionized water to a final volume of 750 mL. 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 yellow dye to aid pipetting) by pouring the total contents of the bottle (60 mL) into a 500-mL graduated cylinder and add distilled or deionized water to a final volume of 300 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 (ready to use): 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. Patient specimens: Dilute each specimen in a recorded proportion with the prediluted 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. This can be prepared in two steps, as follows: dilute 10 µL of serum 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. Reassay of out-of-range samples at lower or higher dilution is rarely necessary. Dilutions lower than 1/10 should not be used.

ASSAY PROCEDURE
(See also schematic overview)

1. Prepare the assay protocol, assigning the appropriate wells for setting up calibrators, diluted patient 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 serum and processed like the other wells.
2. Pipette 100 µL volumes of each calibrator, 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 optical densities (OD) or 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 of assay procedure



CALCULATION OF RESULTS

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

Out-of-line points for individual calibrators: One or more individual calibrators may give anomalous OD 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 R^2 value. 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.

QUALITY CONTROL

Laboratories intending to perform repeated assays should establish their own high-reading and low-reading control sera or urine, stored in small (e.g. 50 µL) aliquots at –70°C or below. An aliquot of each should be thawed and tested in each assay and a record kept of successive results. This serves as a control of test performance, test integrity and operator reliability. The results should be examined for drift (tendency for successive results to rise or fall) or significant deviation from the mean of previous results. Values not deviating by more than 20% from the mean of previous values can be taken to indicate acceptability of the assay. Aliquots of control serum should not be refrozen for repeated assay once thawed, and if a further assay is performed, fresh control aliquots and fresh dilutions of patient specimens should be used.

EXPECTED RESULTS

The mean NGAL concentration in samples from healthy donors was 63 ng/mL (range 37–106 ng/mL, n = 80) in EDTA plasma and 5.3 ng/mL (range 0.7–9.8 ng/mL, n = 7) in urine. In unselected patients admitted to intensive care, the NGAL concentrations in urine ranged from 110 ng/mL to 40,000 ng/mL (40 µg/mL) in urine (n = 11) and from 66 ng/mL to 922 ng/mL in serum (n = 11).

PERFORMANCE CHARACTERISTICS

Limit of detection: The lowest concentration of NGAL giving an OD reading greater than 2 SD above the mean zero (calibrator 1) reading (n = 6) was determined 4 times by different operators, the results ranging from 0.5 to 4.0 pg/mL. These were significantly less than the value of calibrator 2 (10 pg/mL).

Precision: Intraassay variation was determined by measurement of NGAL in a urine pool and a plasma sample with 6 replicates in 4 separate assays performed by 4 different operators. The following results were obtained (CV = coefficient of variation):

Samples	CV Median (range)
Urine	2.1% (1.3-4.0)
Plasma	3.0% (1.2-4.0)

Interassay variation was determined by measurement of NGAL in 5 diluted urine samples and 5 diluted serum samples with 2 replicates in 4 separate assays, performed by 4 different operators. The following results were obtained:

Samples	CV Median (range)
Urine	9.1% (6.8-18.1)
Plasma	8.2% (2.2-11.2)

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

Sample	Measured	Expected	Recovery
Urine	414 ng/mL	410 ng/mL	101%
Serum	399 ng/mL	396 ng/mL	101%
Plasma	441 ng/mL	449 ng/mL	98%

Linearity: NGAL was measured in serial dilutions (n = 5) of a urine sample and a plasma sample. The CV of the mean of the measured values corrected for the dilution was 7.4% for urine and 7.7% for plasma, demonstrating satisfactory linearity in the assay.

Sample material: Analyses of samples of serum, EDTA plasma, citrate plasma, heparin plasma or urine showed no significant differences in analytical recovery, linearity or precision. However, NGAL concentrations are often slightly higher in serum than in plasma,[16] probably because of release from neutrophils during blood clotting.

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 postnuclear supernatant from human neutrophils.[25]

LIABILITY

This kit is only intended for the *in vitro* determination of NGAL in human serum or plasma.

The kit is only intended for use by qualified personnel carrying out research activities.

If the recipient of this kit passes it on in any way to a third party, this instruction must be enclosed, and said recipient shall at own risk secure in favor of BioPorto Diagnostics A/S all limitations of liability herein.

BioPorto Diagnostics A/S shall not be responsible for any damages or loses due to using the kit in any way other than as expressly stated in these Instructions.

The liability of BioPorto Diagnostics A/S shall in no event exceed the commercial value of the kit.

BioPorto Diagnostics A/S shall under no circumstances be liable for indirect, special or consequential damages, including but not limited to loss of profit.

Revision: NG2007-12-EN

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