

NGAL Rapid ELISA Kit

EN

For Research Use Only
Not for use in diagnostic procedures

KIT 037



BIOPORTO[®]
Diagnostics

Revision: NR2012-04RUO**Please read these instructions carefully****APPLICATION**

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. Not for use in diagnostic procedures.

INTRODUCTION

NGAL (neutrophil gelatinase-associated lipocalin) was first discovered in 1989 and, as its name implies, belongs to the lipocalin family of proteins. These are typically small secreted proteins characterized by their ability to bind 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 was fully characterized and named in 1993, but has many synonyms: NL (neutrophil lipocalin; HNL: human NL), lipocalin 2, siderocalin, oncogene protein 24p3 or uterocalin (in the mouse) and neu-related lipocalin or 25-kDa α -microglobulin-related protein (in the rat). Human NGAL consists of a single disulfide-bridged polypeptide chain of 178 amino acid residues with a calculated molecular mass of 21 kDa, but glycosylation increases its apparent molecular mass to 25 kDa. In neutrophils (neutrophilic polymorphonuclear leukocytes) it occurs in monomer and homodimer forms with a small percentage of higher molecular weight forms, and some of it is found in complex with 92-kDa human neutrophil type IV collagenase (gelatinase B or matrix metalloproteinase-9, MMP-9).


PRINCIPLE OF THE ASSAY PROCEDURE

The assay is an ELISA performed in microwells coated with a monoclonal antibody to human NGAL. Bound NGAL is detected with a horseradish peroxidase (HRP)-conjugated monoclonal detection antibody and the assay is developed by incubation with a color-forming substrate. The assay is a rapid 2-step procedure:


Step 1. Aliquots of calibrators, diluted samples and any controls are incubated with HRP-conjugated

detection antibody in the coated microwells. Only NGAL will bind to both coat and detection antibody, while unbound materials are removed by washing.


Step 2. A chromogenic peroxidase substrate containing tetramethylbenzidine (TMB) is added to each test well. The HRP linked to the bound detection antibody 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.

PRINCIPLE OF THE ASSAY PROCEDURE

NGAL antibody-coated wells


The analytical microwells are pre-coated with NGAL capture antibody and are ready to use.


HRP-conjugated NGAL anti-body


HRP-conjugated detection antibody is added to each well.


Samples and calibrators


Calibrators and diluted samples are added to the wells and incubated.


TMB Substrate


Substrate is added to each well and developed.

Stop Solution

Stop Solution is added to each well. Quantitative results are obtained by measuring the absorbance at 450 nm.

KIT COMPONENTS

Item	Contents	Quantity
①	12 x 8 coated Microwells + Frame	96 wells
②	5x Sample Diluent Conc.	1 x 60 mL
③-⑤	NGAL Rapid Calibrator 1-6 0, 0.2, 2, 5, 10, 20 ng/mL.	6 x 1 mL
④	25x Wash Solution Conc.	1 x 30 mL
⑤	HRP-conjugated NGAL Antibody	1 x 6 mL
⑥	TMB Substrate	1 x 12 mL
⑦	Stop Solution	1 x 16 mL
⑧	Polypropylene U-Microwell Plate	96 wells

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

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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 Stop Solution contains 0.5 mol/L 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.
8. Do not interchange components from kits with different batch numbers. The components have been standardized as a unit for a given batch.
9. Hemolyzed, hyperlipemic, heat-treated or contaminated specimens may give erroneous results.
10. Do not dilute specimens directly in the coated microwells.
11. Do not touch or scrape the bottom of the coated microwells when pipetting or aspirating fluid.
12. 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 reagents before the expiry date on the kit box label.
3. Diluted Wash Solution Concentrate remains stable for 4 weeks at 2-8°C. If not all wells are to be used, dilute only the portion of Wash Solution Concentrate required.
4. Diluted Sample Diluent Concentrate remains stable for 24 hours at 2-8°C. If not all wells are to be used, 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 2, 4 and 5.

Determination of NGAL in a single specimen requires 10 µL of fluid sample. Blood specimens should be collected aseptically into EDTA tubes or plain tubes by qualified staff using approved venipuncture techniques. Plasma or serum should be prepared by standard techniques for clinical laboratory testing. Urine should be centrifuged. Cap the prepared specimens. If the assay cannot be performed within 24 hours or specimens are to be shipped, freeze the specimens at -20°C or below. For long-term storage of 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

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 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 12 wells for the 6 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 after use. If not all the wells are to be used, dilute only the required volume of Wash Solution Concentrate with 24 volumes of water to produce a 1/25 dilution.
4. Sample Diluent: Dilute the 5x Sample Diluent Concentrate (contains red 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 after use. If not all the wells are to be used, dilute only the required volume of Sample Diluent Concentrate with 4 volumes of water to produce a 1/5 dilution.
5. NGAL Rapid Calibrators (contains red dye to aid pipetting): The assigned concentrations are indicated on their labels. Do not dilute further.
6. HRP-conjugated NGAL Antibody (ready to use): Do not dilute further.
7. TMB Substrate (ready to use): Do not dilute further.
8. Stop Solution (ready to use): Do not dilute further.

PREPARATION OF SAMPLES

Initial screening dilutions of 1/100 for plasma or serum and 1/50 for urine are recommended. A 1/100 dilution can be prepared by diluting 10 μ L of sample in 990 μ L of Sample Diluent and a 1/50 dilution can be prepared by diluting 10 μ L of sample in 490 μ L of Sample Diluent. Dilutions are mixed by inversion or moderate vortexing. Re-assay of out-of-range samples at lower or higher dilution may be necessary. Dilutions lower than 1/25 should not be used.

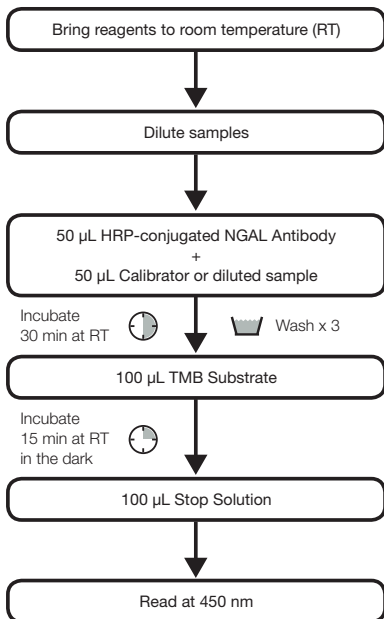
ASSAY PROCEDURE

1. Prepare the assay protocol, assigning the appropriate wells for setting up calibrators, 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 50 μ L of Sample Diluent instead of diluted sample and processed like the other wells.
2. Dilute samples according to the expected NGAL concentrations (1/100 for plasma or serum and 1/50 for urine will be suitable for most samples).
3. Pipette a sufficient volume of each calibrator, each diluted sample and any internal laboratory controls into the appropriate wells of the polypropylene U-microwell plate to permit subsequent transfer of 50 μ L volumes to corresponding coated microwells.
4. Pipette 50 μ L volumes of HRP-conjugated NGAL Antibody into the corresponding positions in the coated microwells. Then with a multichannel pipette rapidly transfer 50 μ L volumes of the calibrator solutions, diluted samples and internal controls from the U-wells into the corresponding coated wells already containing the detection antibody. This method of sample addition is recommended to reduce the difference in incubation time between the first and last samples added to the coated microwells.
5. Cover the wells and incubate for **30 minutes** at

room temperature on a shaking platform set at 200/minute.

6. Aspirate the contents of the microwells and wash the microwells three times with at least 300 μL of the previously diluted Wash Solution. If washing is performed 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. The vigor with which diluted Wash Solution is filled into or emptied from the wells influences final color development. Manual pipetting, which may be very gentle and lead to high color development, is only recommended in the absence of alternatives such as filling the wells by immersion, using a multi-channel manual washing dispenser, or using an automatic washing apparatus.
7. 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 15 minutes** at room temperature in the dark. Start the clock when filling the first well.
8. Add 100 μL of Stop Solution (ready to use) to each well, maintaining the same pipetting sequence and rate as in Step 7. Mix by gentle shaking for 20 seconds, avoiding splashing. Read the wells within 30 minutes.
9. Read the absorbances of the wells at 450 nm in an appropriate microplate reader (reference wavelength 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 mean of duplicate absorbance values for each NGAL Rapid Calibrator on the y-axis against the corresponding NGAL concentrations in ng/mL on the x-axis. The calibration curve must meet the validation requirements. The NGAL concentration of each diluted sample is then found by locating the point on the curve corresponding to the mean of duplicate absorbance values for the diluted sample and reading its corresponding concentration in ng/mL from the x-axis. The concentration of NGAL in the undiluted specimen is calculated by multiplying this result by the sample dilution factor.

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 concentration values between points when the curve is slightly convex to the left, which is the typical finding. 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 NGAL Rapid Calibrator 6 or below that for the NGAL Rapid Calibrator 2 are out of the range of the assay and their concentrations should be noted as >20 ng/mL and <0.2 ng/mL, respectively. The corresponding concentrations in the undiluted samples are calculated $>(20 \times \text{dilution factor})$ ng/mL and $<(0.2 \times \text{dilution factor})$ ng/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 1/25 should not be used.

VALIDATION OF CALIBRATION CURVE

The mean absorbance for the NGAL Rapid Calibrator 6 should be >1.5 . The mean absorbance for any NGAL Rapid Calibrator should be higher than that for the previous NGAL Rapid calibrator, e.g. absorbance(NGAL Rapid Calibrator 6) $>$ absorbance(NGAL Rapid Calibrator 5). The curve should be slightly convex to the left when the results are plotted on linear axes.

CALIBRATION TROUBLESHOOTING

In some circumstances (e.g. high ambient temperature, gentle washing technique) the absorbance values of the calibration curve may be generally elevated, so that the curve flattens off at the higher concentrations giving values near the upper reading range of the microplate reader. This can be compensated for by re-reading all the wells at 405 nm, giving rise to lower absorbance values within the more linear response range of the reader. Results are then calculated from the readings made at the 405 nm wavelength. This avoids having to repeat the assay, but because wells should be read within 30 minutes of adding Stop Solution, it is recommended that the initial readings obtained at 450 nm should be examined within this time period to see whether it is necessary to re-read the plate at 405 nm. If it is known that local laboratory conditions regularly give rise to high calibration curves, the problem can also be overcome by shortening the color development time to 10 or even 5 minutes.

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

QUALITY CONTROL

Laboratories intending to perform repeated assays should establish their own high-reading and low-reading control samples, stored in small (e.g. 50 μ L) 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 controls should not be refrozen for repeated assay once thawed, and if a further assay is performed, fresh control aliquots and fresh dilutions of specimens should be used.

LIABILITY

This ELISA Kit is only intended for the *in vitro* determination of human NGAL. The ELISA Kit is only intended for use by qualified personnel carrying out research 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



Catalogue number



Batch code



Consult instructions for use



Use by



Manufacturer



Keep away from sunlight



Temperature limitation



Do not reuse



Caution, consult accompanying documents



Biological risk



Do not use if package is damaged

WASH SOLUTION 25X

Concentrated Wash Solution.
Dilute before use.

SAMPLE DILUENT 5X

Concentrated Sample
Diluent. Dilute before use.



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