Rat NGAL ELISA Kit

EN

KIT 046

BioPorto® Diagnostics

Please read these instructions carefully

INTENDED USE

For the *in-vitro* determination of rat NGAL in urine, plasma or serum, tissue extracts or culture media. For research use only.

INTRODUCTION

NGAL (neutrophil gelatinase-associated lipocalin)[1] belongs to the lipocalin family of proteins[2]. These are secreted proteins characterized by i) their ability to bind small hydrophobic molecules in a structurally conserved pocket formed by β-pleated sheet, ii) to bind to specific cell-surface receptors, and iii) to form macromolecular complexes. NGAL has many synonyms: perhaps the most widely used is lipocalin 2 (LCN 2); more recently the name siderocalin has been used to express NGAL's ability to bind bacterial siderophores[3]. In the rat, NGAL was first named 25 kDa α_2-microglobulin-related protein[4], and later *neu*-related lipocalin (NRL)[5] because of its expression in mammary tumors initiated by the *neu* (HER2/c-erbB-2) oncogene. In the mouse, the homologous protein is known as oncogene protein 24p3[6], 24-kDa superinducible protein (SIP24) or uterocalin[7].

Rat NGAL consists of a single disulfide-bridged polypeptide chain of 178 amino-acid residues with a calculated molecular mass of 20.5 kDa, but glycosylation increases its apparent molecular mass to 25 kDa. In some situations NGAL may be co-expressed and form complexes with matrix metalloproteinase-9 (MMP-9; also called gelatinase B in human neutrophils, hence NGAL's name).

In the adult rat, NGAL is expressed in the liver, prostate[4], kidney, mammary gland and epithelial cells of the respiratory and alimentary tracts[8]. It may be expressed in additional cell types during embryonic development and in response to inflammatory mediators[9]. NGAL expression in hepatocytes is increased by exposure to IL-1β but not to IL-6[10].

Molecular forms of rat NGAL have been studied in vascular smooth muscle cells[11]. In these cells, NGAL and MMP-9 are co-synthesized in response to angioplastic injury or stimulation with IL-1β. Rat NGAL was shown by immunoblotting to occur in monomer, dimer and trimer forms, as well as a 150-kDa form. MMP-9 occurred in monomer and dimer forms. While complexes of rat NGAL and MMP-9 could not be demonstrated in lysed intimal smooth muscle cells, NGAL/MMP-9 complexes of approximately 115 and 125 kDa were detected in the culture medium, demonstrating the extracellular but not intracellular formation of NGAL/MMP-9 complexes. Rat NGAL has no cysteinyl residue additional to the two forming the intra-chain disulfide bridge, so any participation of NGAL cysteinyl residues in self-association or complex formation with MMP-9 is a matter for further study.

NGAL and acute kidney injury. Apart from the up-regulation of rat NGAL that may occur in malignant transformation or the exposure of certain cell types to inflammatory stimuli, NGAL undergoes an early and dramatic up-regulation in rat proximal tubule cells after ischemia-reperfusion injury to the kidney[12]. These early results have been confirmed[13] and extended to acute nephrotoxic injury by several research groups[14-16]. However, most studies have exclusively measured NGAL up-regulation in terms of mRNA in renal tissue and there is a paucity of quantitative data on NGAL protein levels in rat urine or serum, whether in the basal state or after acute kidney injury. There are considerable differences in the time courses and magnitudes of NGAL mRNA responses to different nephrotoxic agents, and there may also be strain differences in the urinary NGAL response to the toxic stimulus. It nevertheless appears that the determination of urinary NGAL, when used with appropriate caution, may become a convenient end-point in rat models of nephrotoxicity.

PRINCIPLE OF THE ASSAY

The assay is a sandwich ELISA performed in microwells coated with a monoclonal antibody against rat 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 four-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 blue color. The enzymatic reaction is stopped by adding dilute sulfuric acid (Stop Solution), which changes the color to yellow. The yellow 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 samples are read.

KIT COMPONENTS

Item	Contents	Quantity
①	Microwell plate, 96 precoated wells	1 plate
②	5x Sample Diluent Conc.	1 x 50 mL
③	Rat NGAL Calibrators 1-8. 0, 4, 10, 20, 40, 100, 200, 400 pg/mL	8 x 1 mL
④	25x Wash Solution Conc.	1 x 40 mL
⑤	Biotinylated Rat 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

Note: Liquid reagents contain preservative and may be harmful 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 2000 µL
3. Tube racks
4. Adjustable 8- or 12-channel micropipette (50-250 µL range) or repeating micropipette (optional)
5. Clean 1 L and 250 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 samples, 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. Dispose of containers and residues safely 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 samples may give erroneous results.
10. Do not dilute samples directly in the microwells.
11. Do not touch or scrape the bottom of the 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.

COLLECTION OF SAMPLES

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

Determination of NGAL in a single sample requires 10 µL of urine, serum or plasma. Blood samples should be collected into plain, EDTA or heparinized tubes by qualified staff using approved techniques. Serum or plasma should be prepared by standard techniques for laboratory testing. Urine should be centrifuged. Cap the prepared samples and freeze them at –20°C or below if they are not to be analyzed within the next 4 hours. For long-term storage of samples, –70°C or below is recommended. **Avoid repeated freezing and thawing.** Do not use hemolyzed, hyperlipemic, heat treated or contaminated samples.

PREPARATION OF REAGENTS AND SAMPLES

1. Bring all samples and reagents to room temperature (20-25°C). Mix samples thoroughly by gentle inversion and if necessary clear visible particulate matter by low-speed centrifugation (discard pellet).
2. Wash Solution: Dilute the 25x Wash Solution Conc. 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 after use.
3. Sample Diluent: Dilute the 5x Sample Diluent Conc. 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 after use.
4. Rat NGAL Calibrators (ready to use): Do not dilute further. The assigned concentration of each calibrator is indicated on its label.
5. Biotinylated Rat NGAL Antibody (ready to use): Do not dilute further.
6. HRP-Streptavidin (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.

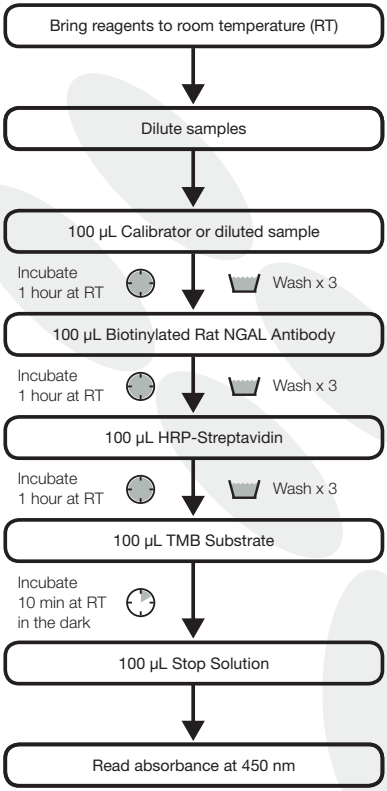
Samples: Dilute each sample in a recorded proportion with the prediluted Sample Diluent to obtain at least 250 µL of diluted solution that can be set up in duplicate wells at 100 µL per well. An initial screening at a dilution of 1/20,000 is recommended for healthy rats. This can be prepared in two steps, as follows: dilute 10 µL of sample in 990 µL of Sample Diluent to make a 1/100 dilution; then dilute 10 µL of the 1/100 dilution in 1990 µL of Sample Diluent to make a 1/20,000 dilution. Dilutions are mixed by inversion or moderate vortexing. Out-of-range samples should be re-assayed at higher or lower dilution as appropriate. Dilutions lower than 1/10 should not be used. If high NGAL levels are expected in samples, the screening dilution should be adjusted accordingly.

ASSAY PROCEDURE

1. Prepare the assay protocol, assigning the appropriate wells for setting up calibrators, diluted samples 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 sample and processed like the other wells.
2. Pipette 100 µL volumes of each calibrator (ready to use), diluted samples and any internal laboratory controls into their 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 them three times with 300 µL diluted Wash Solution. If the 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 Rat 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 set at 200/minute.
5. Wash as described above in Step 3.
6. Dispense 100 µL of HRP-Streptavidin (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 set at 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. Start the clock when filling the first well. Cover the wells and incubate **for exactly 10 minutes** at room temperature in the dark.

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 absorbance values 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

This procedure can be performed manually using graph paper with linear x and y axes. NGAL concentrations are drawn and read on the x-axis and the absorbance values on the y-axis. A smooth curve can be drawn through the points obtained for the calibration curve, or adjacent points on the curve can be joined by straight lines. The latter procedure may slightly overestimate or underestimate concentration values between points when the curve is slightly convex to left or 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.

Results can also be calculated by means of 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 Rat NGAL Calibrator 8 or below that for the Rat NGAL Calibrator 2 are out of the range of the assay and their concentrations should be noted as >400 pg/mL and <4 pg/mL, respectively. The corresponding concentrations of the undiluted samples are calculated as >(400 x dilution factor) pg/mL and <(4 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.

VALIDATION OF CALIBRATION CURVE

The mean absorbance value for the 400 pg/mL Rat NGAL Calibrator should be >1.5. The mean absorbance value for any Rat NGAL Calibrator should be higher than that for the next lower calibrator, e.g. calibrator 7 (200 pg/mL) should give a higher reading than calibrator 6 (100 pg/mL). 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 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.

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-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 samples should not be refrozen for repeated assay once thawed, and if a further assay is performed, fresh control aliquots and fresh dilutions of samples should be used.

EXPECTED RESULTS

Absolute concentrations of NGAL in rat urine, serum or plasma are not known, as results obtained by immunochemical techniques have not been standardized against an accepted purified preparation of rat NGAL of known gravimetric concentration. Normal values have yet to be assigned to urine, plasma and serum concentrations of NGAL in different rat strains. Preliminary determinations in healthy Sprague Dawley rats (n = 5) gave mean values of 1.8 µg/mL in urine and 0.6 µg/mL in EDTA plasma, while corresponding values for Wistar Hannover rats (n = 5) were 0.9 µg/mL and 0.2 µg/mL.

The calibrators are dilutions of recombinant full-length rat NGAL produced in *E. coli*. The concentration of purified calibrator material was determined by absorbance measurement at 280 nm using an extinction coefficient (30,000 $M^{-1}cm^{-1}$) calculated on the basis of the amino-acid composition of rat NGAL.

PERFORMANCE CHARACTERISTICS

Limit of detection: The lowest concentration of rat NGAL giving an absorbance reading greater than 2SD above the mean zero (Calibrator 1) reading (n = 20) was determined to 0.5 pg/mL. This was significantly lower than the value of Calibrator 2 (4 pg/mL).

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

Samples	CV
Urine 1	3%
Urine 2	3%
Plasma 1	3%
Plasma 2	4%

Interassay variation was determined by measuring rat NGAL in six diluted urine samples and five diluted EDTA plasma samples with 2 replicates in 2-4 separate assays. The following results were obtained:

Samples	Median CV (range)
Urine	10% (5%-19%)
Plasma	12% (4%-20%)

Analytical recovery: Urine and EDTA plasma samples were spiked with four different concentrations of calibrator material and analyzed in the assay. Recovery was calculated from (measured value)/(expected value) expressed as a percentage.

Sample	Measured	Expected	Recovery
Urine 1	61 pg/mL	55 pg/mL	111%
Urine 2	101 pg/mL	93 pg/mL	109%
Urine 3	183 pg/mL	179 pg/mL	102%
Urine 4	350 pg/mL	346 pg/mL	101%
Plasma 1	54 pg/mL	50 pg/mL	108%
Plasma 2	92 pg/mL	88 pg/mL	105%
Plasma 3	173 pg/mL	174 pg/mL	99%
Plasma 4	349 pg/mL	341 pg/mL	102%

Linearity: Rat NGAL was measured in serial dilutions (n = 8) of two urine samples and two EDTA plasma samples. The CV of the mean of the measured values corrected for the dilution was 5% and 4% for urine samples 1 and 2, respectively, and 5% and 4% for EDTA plasma samples 1 and 2, respectively, demonstrating satisfactory linearity of the assay.

Specificity: The two monoclonal antibodies used in this assay were raised against recombinant rat NGAL and react with 25-kDa peaks of recombinant rat NGAL and native NGAL from rat urine and serum subjected to molecular size exclusion chromatography.

LIABILITY

This kit is only intended for the *in-vitro* determination of rat NGAL in urine, plasma or serum, tissue extracts or culture media.

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

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 losses 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: SN2010-12-EN

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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

Concentrated Sample Solution.
Dilute before use.

Concentrated Wash Solution.
Dilute before use.

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