



ELISA PRODUCT INFORMATION & MANUAL

Monkey Lipocalin-2/NGAL *NBP2-62164*

Enzyme-linked Immunosorbent Assay for quantitative
detection of Monkey Lipocalin-2/NGAL.

For research use only.

Not for diagnostic or therapeutic procedures.

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Novus kits are guaranteed for 6 months from date of receipt

Please read these instructions carefully**INTENDED USE**

For the *in-vitro* determination of NGAL from Rhesus (*Macaca mulatta*) or Cynomolgus (*Macaca fascicularis*) monkeys in urine, plasma, serum, tissue extracts or culture media. For research use only.

INTRODUCTION

NGAL (neutrophil gelatinase-associated lipocalin)¹ belongs to the lipocalin family of proteins². These are secreted proteins characterized by their ability i) to bind small hydrophobic molecules in a structurally conserved pocket formed by 8-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 certain bacterial siderophores³.

The NCBI reference sequence XP_001083008.1 from genomic sequencing of *Macaca mulatta* (chromosome 15) suggests that Rhesus monkey NGAL consists of a single disulfide-bridged polypeptide chain of 178 amino-acid residues with a calculated molecular mass of 20.4 kDa. By analogy with NGAL from other mammalian species, glycosylation would be expected to increase its apparent molecular mass on SDS-PAGE to about 24-25 kDa. The N-glycosylation site at residue 65 is intact and is in fact conserved in all known NGAL sequences from placental mammals.

Because Rhesus monkey NGAL has not yet been isolated and no antibodies or immunochemical detection methods have hitherto been developed to detect or measure it, there are no published data on the physiological or pathological functions of this protein. However, it is likely that some features of the biosynthesis, location and release of Rhesus monkey NGAL will be similar to those of its homologues in other species, particularly man. The present kit has in fact been developed to extend NGAL research and the use of NGAL as a marker molecule, especially for kidney injury, to the Rhesus and closely related Cyn-

omolgus monkey. In the following, some pathophysiological features of NGAL in humans and other mammalian species will be mentioned, because these two monkey species may show similar features.

In some situations, NGAL may be co-expressed with matrix metalloproteinase-9 (MMP-9)^{1,4}, but whether Rhesus monkey NGAL forms a complex with this enzyme is unknown, given that, unlike its human homologue, it does not possess a third cysteinyl residue capable of forming an intermolecular disulfide bridge with the enzyme.

In other mammals, NGAL is expressed in neutrophil polymorphonuclear leucocytes¹ and at lower basal levels in various other cell types, tissues or organs. These include epithelial cells of the uterus, mammary gland, lung, spleen, vagina and epididymis^{5,6}. It may be expressed in additional cell types during embryonic development and in response to various stimuli. This applies to certain kidney cells (see below), LPS-stimulated macrophages⁷, dexamethasone-stimulated L-cells⁸, and fibroblasts stimulated with basic fibroblast growth factor⁹. In the mouse, NGAL (often called 24p3 or uterocalin) is a type-1 acute-phase protein, being secreted by hepatocytes in the *in-vivo* turpentine model or in response to TNF- α or dexamethasone *in vitro*¹⁰.

In disease, NGAL may be moderately raised in bacterial infections because of release from activated neutrophils¹¹ or from epithelial cells¹². It may also be moderately raised in atheroma⁴ and the ailing myocardium¹³, and in certain epithelial cancers such as those of the mammary gland¹⁴, gastrointestinal tract¹⁵ or urothelium¹⁶.

NGAL and acute kidney injury

Apart from the expression of mammalian NGAL that occurs in the above situations, NGAL was found to undergo an early and dramatic upregulation in mouse kidney cells after infection with SV40 or polyoma virus¹⁷. This was the first indication of NGAL's early response to kidney cell injury.

Years later this finding was extended to post-ischemic¹⁸ and nephrotoxic injury¹⁹. The marked upregulation of NGAL mRNA and protein levels in the early post-ischemic rat kidney was detected pre-

dominantly in proximal tubule cells¹⁸ and NGAL was easily detected by Western blotting of urine after ischemia and cisplatin-induced nephrotoxicity²⁰.

Urinary NGAL has also been found to be raised in mouse models of diabetic and obstructive nephropathy²¹. However, there is a paucity of quantitative data on NGAL levels in non-human urine, plasma or serum, whether in the basal state or after kidney injury, and there are as yet no published data from any monkey species. It is the purpose of the present kit to allow such data to be obtained.

In man, it is now widely recognized that NGAL, whether measured in urine or plasma, is the earliest responding marker of acute kidney injury known, with the further advantage that its response is high and easy to measure while retaining proportionality with the severity of injury (see e.g. ref.²²). It is therefore to be hoped that the determination of urinary or plasma NGAL may become a convenient end-point in Rhesus and Cynomolgus monkey models of renal injury, nephrotoxicity and other nephropathies.

In the following instructions, the unqualified term "monkey" refers to Rhesus and/or Cynomolgus monkeys.

PRINCIPLE OF THE ASSAY

The assay is a sandwich ELISA performed in microwells coated with a mouse monoclonal antibody against monkey NGAL. Bound NGAL is detected with another mouse 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 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 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	12 x 8 pre-coated microwells + frame	96 wells
②	5x Sample Diluent Conc.	1 x 50 mL
③	Monkey NGAL Calibrator Stock 1000 pg/mL	3 x 1 mL
④	25x Wash Solution Conc.	1 x 40 mL
⑤	Biotinylated Monkey-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

- Adjustable micropipettes covering the range 1-1000 μ L and corresponding disposable pipette tips
- Polypropylene tubes to contain up to 2000 μ L

3. Tube racks
4. Adjustable 8- or 12-channel micropipette (50-250 μ L 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
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. Unopened materials/reagents remain stable until 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 Conc. remains stable for 24 hours at 2-8°C. If not using all wells, dilute only the portion of Wash Solution Conc. required.
5. Diluted Sample Diluent Conc. remains stable for 24 hours at 2-8°C. If not using all wells, dilute only the portion of Sample Diluent Conc. 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.

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.

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 μ L of urine, serum or plasma. Blood samples should be collected into EDTA, heparinized or plain tubes by qualified staff using approved techniques. Plasma or serum should be prepared by standard techniques for laboratory testing. Urine should be centrifuged to remove cellular debris. 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.

Tube no.	Vol. of Sample Diluent (μL)	Vol. of Monkey NGAL Calibrator Stock 1000 pg/mL (μL)	Final volume* (μL)	Dilution factor	Monkey NGAL concentration (pg/mL)
1	200	200	400	2	500
2	300	100	400	4	250
3	360	40	400	10	100
4	380	20	400	20	50
5	390	10	400	40	25
6	990	10	1000	100	10
7	400	-	400	-	0

*Only 200 μL are used for setting up the assay, 100 μL being added to each of duplicate wells.

Avoid repeated freezing and thawing. Do not use hemolyzed, hyperlipemic, heat-treated or contaminated samples.

PREPARATION OF REAGENTS AND SAMPLES

- 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).
- 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 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.
- 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. If

not all the wells are to be used, dilute (1/25) only the volume of Wash Solution Conc. required.

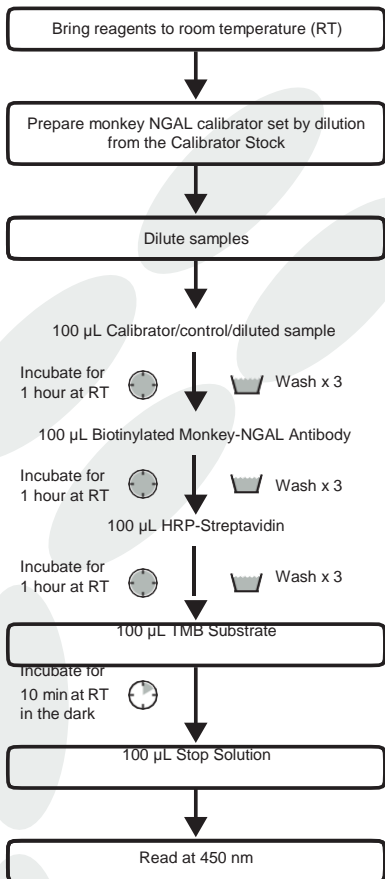
- Sample Diluent: Dilute the 5x Sample Diluent Conc. by pouring the total contents of the bottle (50 mL) into a 250-mL graduated cylinder and add distilled or deionized water to a final volume of 250 mL. Mix thoroughly and store at 2-8°C after use. If not all the wells are to be used, dilute (1/5) only the volume of Sample Diluent Conc. required.
- Calibrator dilutions: Dilute the Monkey NGAL Calibrator Stock (1000 pg/mL) manually as shown in the Table by adding 200, 100, 40, 20, 10 and 10 μL of the 1000 pg/mL calibrator stock to tubes 1-6, respectively. Tube 7 serves as the zero calibrator, consisting of prediluted Sample Diluent alone. This procedure will result in eight calibrators of different monkey NGAL concentrations: the calibrator stock of 1000 pg/mL plus tubes 1-7 containing 500, 250, 100, 50, 25, 10 and 0 pg/mL, respectively.
- Biotinylated Monkey-NGAL Antibody (ready to use): Do not dilute further.
- HRP-Streptavidin conjugate (ready to use): Do not dilute further.
- TMB Substrate (ready to use): Do not dilute further.
- Stop Solution (ready to use): Do not dilute further.
- Samples: Dilute each sample in a recorded pro-

portion with the pre-diluted Sample Diluent to obtain at least 250 μL of diluted sample solution that can be set up in duplicate wells at 100 μL per well. An initial screening at a dilution of 1/250 is recommended. This can be prepared in two steps, as follows: dilute 10 μL of sample in 490 μL of pre-diluted Sample Diluent to make a 1/50 dilution; then dilute 50 μL of the 1/50 dilution in 200 μL of pre-diluted Sample Diluent to make 250 μL of a 1/250 dilution. Dilutions are mixed by inversion or moderate vortexing. Out-of-range samples should be re-assayed at higher or lower dilution as appropriate.

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 pre-diluted Sample Diluent instead of diluted sample and processed like the other wells.
2. Pipette 100 μL volumes of each calibrator dilution, 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 the wells three times with 300 μL of 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 Monkey-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 highest Monkey NGAL Calibrator or below that for the lowest Monkey NGAL Calibrator above zero 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 of the undiluted samples are calculated 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.

VALIDATION OF CALIBRATION CURVE

The mean absorbance value for the 1000 pg/mL Monkey NGAL Calibrator should be >1.5. The mean absorbance value for any Monkey NGAL Calibrator should be higher than that for the next lower calibrator, e.g. the 500 pg/mL calibrator should give a higher reading than the 250 pg/mL calibrator. 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.

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 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 monkey urine or serum are not known, and any results that may have been obtained by immunochemical techniques have not been standardized to an accepted purified preparation of monkey NGAL of known gravimetric concentration. Normal values have yet to be assigned to urine, plasma and serum concentrations of NGAL in different monkey species.

PERFORMANCE CHARACTERISTICS

Limit of detection: The lowest concentration of monkey NGAL giving an absorbance reading greater than 2 SD above the mean zero (calibrator 1) reading was 1.5 pg/mL, which is significantly lower than the value of the lowest calibrator concentration above zero (10 pg/mL).

Intraassay (within-run) and interassay (between-run) reproducibility: Dilutions of two monkey urine samples (U1, U2) and two monkey plasma samples (P1, P2) were run in 8 replicates for determining within-run reproducibility and in replicates in 3-4 assays on different days for determining between-run reproducibility. The following results were obtained (CV = coefficient of variation):

Sample	Mean NGAL conc. (diluted, pg/mL)	CV within-run	CV between-run
U1	825	4%	8%
U2	245	4%	4%
P1	82	3%	5%
P2	31	4%	10%

Analytical recovery: Four different dilutions of urine (U1-4) and plasma (P1-4) were spiked with calibrator material and analyzed in the assay after appropriate further dilution. Recovery was calculated as ("Measured"/"Calculated") x 100%:

Sample	Measured (pg/mL)	Calculated (pg/mL)	Recovery
U1	139	143	97%
U2	236	240	98%
U3	439	447	98%
U4	791	856	92%
Mean recovery (U)			96%
P1	137	139	99%
P2	239	236	101%
P3	425	443	96%
P4	786	852	92%
Mean recovery (P)			97%

Linearity: Monkey NGAL was measured in serial dilutions ($n = 8$) of two urine samples and two plasma samples. The CV of the mean of the measured values corrected for the dilution was 4% and 1% for the urine samples respectively, and 4% and 3% for the plasma samples respectively, demonstrating a satisfactory linearity of the assay.

Specificity: The two mouse monoclonal antibodies used in this assay were raised against recombinant Rhesus monkey NGAL and react with urine and serum samples from both Rhesus and Cynomolgus monkeys, but not from the marmoset (*Callithrix jacchus*). Assay of fractions from molecular size exclusion chromatography of Cynomolgus monkey serum showed a peak of immunoreactivity at an elution volume compatible with the expected molecular size of NGAL.

LIABILITY

For *in-vitro* research use only.

This ELISA Kit is only intended for the *in vitro* determination of Rhesus or Cynomolgus monkey 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 Novus Biologicals all limitations of liability herein.

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Related products

Cat. No.	Product name
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