Human NGAL
ELISA Kit

REF KIT 036RUO

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bioporto
D I A G N O S T I C S

Please read these instructions carefully

INTENDED USE

An enzyme-linked immunosorbent assay for quantitative determination of human NGAL in urine and EDTA plasma. For Research Use Only.

INTRODUCTION

NGAL is a biomarker of kidney tubular damage and a biological response to the damage process which is distinctly separated from loss of kidney function called Acute Kidney Injury (AKI)[1]. As damage often precedes loss of function renal expression of NGAL increases dramatically in a variety of causes and NGAL is released into both urine and plasma. Accordingly, renal disorders with presence of acute tubular damage are associated with raised urinary and plasma NGAL levels. Urinary NGAL serves as an early marker of acute kidney injury after cardiopulmonary bypass surgery[2,3] and both urinary and plasma NGAL levels provide an early indication of acute renal injury in unselected patients in intensive care[4]. Raised urinary and plasma levels of NGAL have also been observed in patients with chronic kidney diseases[5], and persistently raised urinary levels after renal transplantation are associated with poor graft function[6].

PRINCIPLE OF THE ASSAY PROCEDURE

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

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

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

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

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

KIT COMPONENTS

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

Note: Liquid reagents contain preservatives with 2-methyl-2H-isothiazol-3-one (MIT) which may cause an allergic skin reaction.

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 microwell plate
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 Research Use Only. Not for use in diagnostic procedures

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 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.
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 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.
16. Wear gloves when handling specimen during collection of specimen, preparation of specimen or procedure.
17. The device contains material of human or animal origin and should be handled as potential carrier and transmitter of disease. The bovine serum albumin is manufactured from USDA-inspected bovine plasma and is validated for prion (TSE) clearance.
18. Do not use the kit if the packaging is damaged.

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

COLLECTION OF SPECIMENS
Handle and dispose of all blood-derived or urine specimens as if they were potentially infectious. See Precautions, sections 1, 2, 4, 5 and 16.
Determination of NGAL in a single specimen requires 10 µL of fluid sample. Blood specimens should be collected aseptically into EDTA tubes by qualified staff using approve venipuncture techniques. Plasma should be prepared by standard techniques for laboratory testing. Urine should be centrifuged. Samples can be kept cool at 2-8°C for two days. For storage of specimens, -70°C or below is recommended. Do not use hemolyzed, hyperlipemic, heat-treated or contaminated specimens.

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

mL) into a 250-mL graduated cylinder and add distilled or deionized water to a final volume of 250 mL. Mix thoroughly and store at 2-8°C. If not all the wells are to be used, dilute (1/5) only the volume of Sample Diluent Concentrate required.

5. NGAL Calibrators (contains red dye to aid pipetting): The assigned concentrations are indicated on their labels. Do not dilute further.

6. Biotinylated NGAL Antibody (ready to use): Do not dilute further.

7. HRP-Streptavidin Conjugate (ready to use): Do not dilute further.

8. TMB Substrate (ready to use): Do not dilute further.

9. Stop Solution (ready to use): Do not dilute further.

10. NGAL Controls Low and High: Dilute each control with the pre-diluted Sample Diluent to obtain at least 250 µL of diluted solution that can be set up in duplicate wells at 100 µL per well. Dilute the controls 1/500. This can be prepared in two steps, as follows: dilute 10 µL of sample in 190 µL of Sample Diluent to make a 1/20 dilution; then dilute 10 µL of the 1/20 dilution in 240 µL of Sample Diluent to make a 1/500 dilution.

11. Specimens: Dilute each specimen in a recorded proportion with the pre-diluted Sample Diluent to obtain at least 250 µL of diluted solution that can be set up in duplicate wells at 100 µL per well. An initial screening at a dilution of 1/500 is recommended for physiological fluids. This can be prepared in two steps, as follows: dilute 10 µL of sample in 190 µL of Sample Diluent to make a 1/20 dilution; then dilute 10 µL of the 1/20 dilution in 240 µL of Sample Diluent to make a 1/500 dilution. Dilutions are mixed by inversion or moderate vortexing. Re-assay of out-of-range samples at lower or higher dilution is rarely necessary. Dilutions lower than 1/10 should not be used.
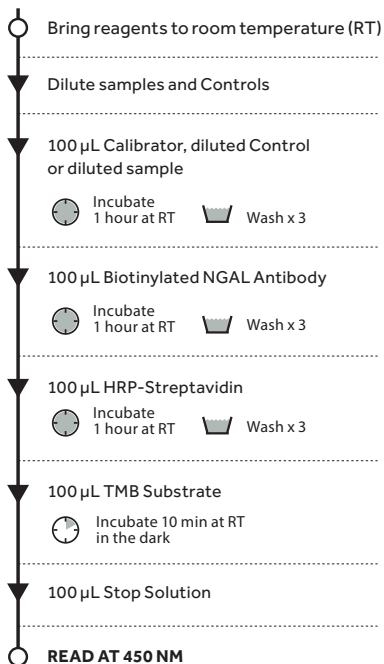
ASSAY PROCEDURE

1. Prepare the assay protocol, assigning the appropriate wells for setting up calibrators, diluted NGAL controls, diluted specimens and any internal laboratory controls in duplicate. If a reference wavelength of 650 or 620 nm is not available on the ELISA reader, a reagent blank well can be assigned. This is set up with 100 µL of Sample Diluent instead of diluted specimen and processed like the other wells.

2. Pipette 100 µL volumes of each calibrator, diluted NGAL controls, diluted specimens and any internal laboratory controls into the corresponding positions in the microwells. Cover the wells and incubate for 60 minutes at room temperature on a shaking platform set at 200/minute.

3. Aspirate the contents of the microwells and wash the microwells three times with 300 µL diluted Wash Solution. If washing is done manually, empty the microwells by inversion and gentle shaking into a suitable container, followed by blotting in the inverted position on a paper towel. A dwell time of 1 minute before emptying is recommended for at least the last wash of the cycle.

4. Dispense 100 µL of Biotinylated NGAL Antibody (ready to use) into each microwell. A multichannel or repeating micropipette can be used. Cover the wells and incubate for 60 minutes at room temperature on a shaking platform (200/minute).

5. Wash as described above in Step 3.

6. Dispense 100 µL of HRP-Streptavidin Conjugate (ready to use) into each microwell. A multichannel or repeating micropipette can be used. Cover the wells and incubate for 60 minutes at room temperature on a shaking platform (200/minute).

7. Wash as described above in Step 3.

8. Dispense 100 µL of TMB Substrate (ready to use) into each microwell. The use of a multichannel micropipette is recommended to reduce pipetting time. Cover the wells and incubate for **exactly 10 minutes** at room temperature in the

dark. Start the clock when filling the first well.

9. Add 100 µL Stop Solution (ready to use) to each well, maintaining the same pipetting sequence and rate as in Step 8. Mix by gentle shaking for 20 seconds, avoiding splashing. Read the wells within 30 minutes.

10. Read the absorbances of the wells at 450 nm in an appropriate microplate reader (reference wave-length 650 or 620 nm). If no reference wavelength is available, the value of the reagent blank well is subtracted from each of the other values before other calculations are performed.

SCHEMATIC OVERVIEW

Bring reagents to room temperature (RT)

Dilute samples and Controls

100 µL Calibrator, diluted Control or diluted sample

Incubate 1 hour at RT   Wash x 3

100 µL Biotinylated NGAL Antibody

Incubate 1 hour at RT   Wash x 3

100 µL HRP-Streptavidin

Incubate 1 hour at RT   Wash x 3

100 µL TMB Substrate

Incubate 10 min at RT in the dark

100 µL Stop Solution

READ AT 450 NM

CALCULATION OF RESULTS

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

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

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

VALIDATION OF CALIBRATION CURVE

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

CALIBRATION TROUBLESHOOTING

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

i) An erroneous singlet or duplicate result should be eliminated from the curve, and the remaining results refitted by the 4-parameter logistic procedure. If a satisfactory fit is obtained, provisional concentration results can be calculated from it.

ii) If no satisfactory fit can be obtained in this way, but the curve is otherwise consistent, provisional results can be obtained from straight lines or simple cubic spline fitting between the means of duplicates, omitting the erroneous point.

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

A deviant result for an individual calibrator can be due to 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

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

LIABILITY

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

REFERENCES

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IFU-0095 v.07 KIT036 IFU RUO

REF	Catalogue number		Temperature limitation
LOT	Batch code		Caution, consult accompanying documents
	Consult instructions for use		Biological risk
	Use by		Do not use if package is damaged
	Manufacturer		Maximum numbers of tests
	Keep away from sunlight		

RELATED PRODUCTS

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

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