Human NGAL ELISA Kit

DPG-2NGAL

Edition 11-12

ELISA KIT
PRODUCT INFORMATION & MANUAL

Read carefully prior to starting procedures!
For use in laboratory research only
Not for clinical or diagnostic use

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1.   INTENDED USE

The human NGAL ELISA kit is to be used for the *in vitro* quantitative determination of human NGAL in plasma, urine and cell culture supernatant samples. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

The analysis should be performed by trained laboratory professionals.

2.   INTRODUCTION

Human neutrophil gelatinase-associated lipocalin (NGAL), also known as human neutrophil lipocalin (HNL, ~25 kDa) is present in specific granules. Neutrophils co-express NGAL and human neutrophil gelatinase B (MMP-9), which are both stored in the same specific granules. Upon activation, human neutrophils secrete the content of these specific granules. NGAL exists as monomer (25 kDa), homodimer, and as heterodimer complex with human neutrophil gelatinase B (MMP-9) (ca 125kDa). NGAL can be secreted as monomer as well as in complex MMP-9.

NGAL is a member of the lipocalin superfamily, a family of small extracellular proteins that are characterized by the ability to bind small hydrophobic molecules, such as retinol. Furthermore, they can bind to specific cell surface receptors. Lipocalins can be used as biochemical markers in relation to various diseases, such as inflammatory diseases, cancer, lipid disorders, liver and kidney function. NGAL has been identified as an iron-transporting protein during nephrogenesis, demonstrating a role for NGAL in the formation of kidney epithelia. Furthermore, NGAL is suggested to have microbicidal properties and to play a role in regulation of inflammation and cellular growth.

NGAL is generally expressed at very low concentrations in a wide range of human tissues, including kidney, trachea, lungs, stomach, and colon. In injured epithelia, NGAL expression is induced and consequently NGAL concentrations are elevated, for example, during acute bacterial infections, asthma and chronic obstructive pulmonary disease. Furthermore, NGAL is strongly expressed in bowel adenomas, adenocarcinomas of the breast and urothelial carcinomas.

Because of its small size, NGAL is readily secreted and detectable in urine, turning NGAL into an early marker for certain renal conditions. Measurement of NGAL is an experimental tool to distinguish between acute viral and bacterial infections. NGAL expression is most extensively studied as biomarker for renal diseases, but many other pathologies are associated with raised levels of urinary or humoral NGAL. However, measurement of NGAL levels in other disorders like, for example, infection must be treated with caution, since bystander neoplastic conditions or renal disorders themselves increase NGAL levels and may therefore distort the measurements and interpretation of the results.

The Diapharma NGAL assay is capable of binding monomeric and dimeric NGAL as well as NGAL/MMP9 complex. In urine, by preference the heterodimeric complex is measured. The assay contains purified recombinant NGAL as a standard.

3.   KIT FEATURES

- Working time of 3½ hours.
- Minimum concentration which can be measured is 0.4 ng/ml.
- Measurable concentration range of 0.4 to 100 ng/ml.
- Working volume of 100 µl/well.

Cross-reactivity

Potential cross-reacting proteins detected in the human NGAL ELISA:

Cross reactant	Reactivity
Bovine	No
Dog	No
Goat	No
Horse	No
Mouse	No
Rabbit	No
Rat	No
Pig	No
Sheep	No

Table 1

Cross-reactivity for other species or proteins/peptides has not been tested.

4.   PROTOCOL OVERVIEW
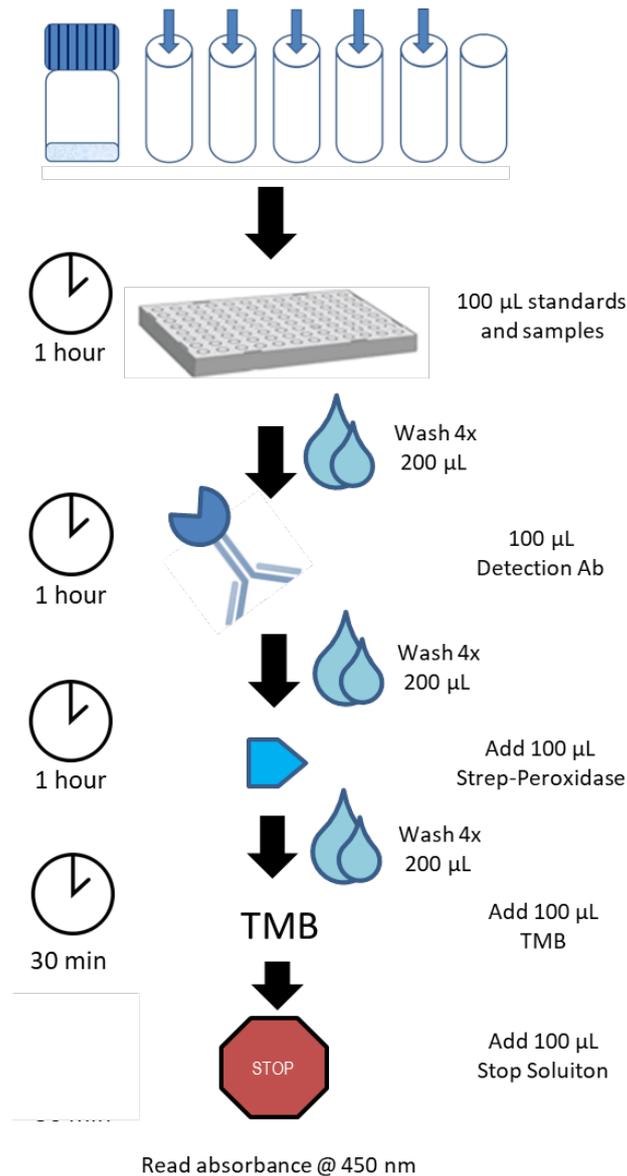
- The human NGAL ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time of 3½ hours.
- The efficient format of a plate with twelve disposable 8-well strips allows free choice of batch size for the assay.
- Samples and standards are incubated in microtiter wells coated with antibodies recognizing human NGAL.
- Biotinylated tracer antibody will bind to captured human NGAL.
- Streptavidin-peroxidase conjugate will bind to the biotinylated tracer antibody.
- Streptavidin-peroxidase conjugate will react with the substrate, tetramethylbenzidine (TMB).
- The enzyme reaction is stopped by the addition of oxalic acid.
- The absorbance at 450 is measured with a spectrophotometer. A standard curve is obtained by plotting the absorbance (linear) versus the corresponding concentrations of the human NGAL standards (log).
- The human NGAL concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.

5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Kit component	2 x 96 well	Color code
Wash buffer 20x	1 vial (60 ml)	Colorless
Dilution buffer 5x	1 vial (30 ml)	Green
Standard	4 vials, lyophilized	White
Tracer, biotinylated	2 vials, 1 ml lyophilized	White
Streptavidin-peroxidase 100x	1 tube, 0.25 ml in solution	Brown
TMB substrate	1 vial (22 ml)	Brown
Stop solution	1 vial (22 ml)	Red
12 Microtiter strips, pre-coated	2 plates	
Certificate of Analysis	1	
Manual	1	

Table 1

- Upon receipt, store individual components at 2 - 8°C. Do not freeze.
- Do not use components beyond the expiration date printed on the kit label.
- The standard and tracer in lyophilized form and the streptavidin-peroxidase in concentrated solution are stable until the expiration date indicated on the kit label, if stored at 2 - 8°C.
- The exact amount of the standard is indicated on the label of the vial and the Certificate of Analysis.
- Once reconstituted the standard should be used within 15 minutes. The standard is single use. After reconstitution the standard cannot be stored.
- Once reconstituted the tracer is stable for 1 month if stored at 2 - 8°C.
- The streptavidin-peroxidase can only be stored in concentrated solution and is not stable when stored diluted.
- Upon receipt, foil pouch around the plate should be vacuum-sealed and unpunctured. Any irregularities to aforementioned conditions may influence plate performance in the assay.
- Return unused strips immediately to the foil pouch containing the desiccant pack and reseal along the entire edge of the zip-seal. Quality guaranteed for 1 month if stored at 2 - 8°C.

Materials required but not provided

- Calibrated micropipettes and disposable tips.
- Distilled or de-ionized water.
- Plate washer: automatic or manual.
- Polypropylene tubes.
- Calibrated ELISA plate reader capable of measuring absorbance at 450 nm.
- Adhesive covers can be ordered separately. Please contact your local distributor.
- Centrifuge for 1 ml tubes.

6.   WARNINGS AND PRECAUTIONS

- For research use only, not for diagnostic or therapeutic use.
- This kit should only be used by qualified laboratory staff.
- Do not add under any circumstances sodium azide as preservative to any of the components.
- Do not use kit components beyond the expiration date.
- Do not mix reagents from different kits and lots. The reagents have been standardized as a unit for a given lot. Use only the reagents supplied by manufacturer.
- The assay has been optimized for the indicated standard range. Do not change the standard range.
- Open vials carefully: vials are under vacuum.
- It is advised to spin down streptavidin-peroxidase tubes before use.
- Do not ingest any of the kit components.
- Kit reagents contain 2-chloroacetamide as a preservative. 2-Chloroacetamide is harmful in contact with skin and toxic if swallowed. In case of accident or if you feel unwell, seek medical advise immediately.
- The TMB substrate is light sensitive, keep away from bright light. The solution should be colorless until use.
- The stop solution contains 2% oxalic acid and can cause irritation or burns to respiratory system, skin and eyes. Direct contact with skin and eyes should be strictly avoided. If contact occurs, rinse immediately with plenty of water and seek medical advise.
- Incubation times, incubation temperature and pipetting volumes other than those specified may give erroneous results.
- Do not reuse microwells or pour reagents back into their bottles once dispensed.
- Handle all biological samples as potentially hazardous and capable of transmitting diseases.
- Hemolyzed, hyperlipemic, heat-treated or contaminated samples may give erroneous results.
- Use polypropylene tubes for preparation of standard and samples. Do not use polystyrene tubes or sample plates.

7.   SAMPLE PREPARATION

Collection and handling

Plasma

Please be aware that human NGAL is released from neutrophils into serum in the process of blood coagulation. This will lead to false positive results. It is therefore advised to use 'careful plasma', which can be obtained as follows.

Keep freshly collected blood on ice. Within 20 minutes after blood sampling, separate plasma by centrifugation: 1500xg at 4°C for 15 min. Remove plasma and transfer to fresh polypropylene tube. Be careful to not disturb white cells in the buffy coat. Recentrifuge the transferred plasma in order to avoid every contamination with white blood cells: 1500xg at 4°C for 15 min.

Most reliable results are obtained if EDTA plasma is used.

Urine

Collect urine using normal aseptic techniques. Centrifuge the urine to remove debris (1500xg at 4 °C for 15 min). Transfer urine to a fresh polypropylene tube.

Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Storage at -20°C can affect recovery of human NGAL. Use samples within 24 hours after thawing. Avoid multiple freeze-thaw cycles which may cause loss of human NGAL activity and give erroneous results.

Do not use hemolyzed, hyperlipemic, heat-treated or contaminated samples.

Before performing the assay, samples should be brought to room temperature (18 – 25°C) and mixed gently. Prepare all samples (controls and test samples) prior to starting the assay procedure. Avoid foaming.

Dilution procedures

Plasma samples

Human NGAL can be measured accurately if plasma samples are diluted at least 10x with supplied dilution buffer in polypropylene tubes.

Note that most reliable results are obtained with EDTA plasma.

Urine samples

Human NGAL can be measured accurately if urine samples are diluted at least 20x with supplied dilution buffer in polypropylene tubes.

Remark regarding recommended sample dilution

The mentioned dilution for samples is a minimum dilution and should be used as a guideline. The recovery of human NGAL from an undiluted sample is not 100% and may vary from sample to sample. When testing less diluted samples it is advisable to run recovery experiments to determine the influence of the matrix on the detection of human NGAL.

Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.

8.   REAGENT PREPARATION

Allow all the reagents to equilibrate to room temperature (20 – 25°C) prior to use. Return to proper storage conditions immediately after use.

Wash buffer

Prepare wash buffer by mixing 60 ml of 20x wash buffer with 1140 ml of distilled or de-ionized water, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of wash buffer by diluting 1 part of the 20x wash buffer with 19 parts of distilled or de-ionized water.

Dilution buffer

Prepare dilution buffer by mixing 30 ml of the 5x dilution buffer with 120 ml of distilled or de-ionized water, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of dilution buffer by diluting 1 part of the 5x dilution buffer with 4 parts of distilled or de-ionized water. Concentrated buffer may contain crystals. In case the crystals do not disappear at room temperature within 1 hour, concentrated dilution buffer can be warmed up to 37°C. Do not shake the solution.

Standard solution

The standard is reconstituted by pipetting the amount of dilution buffer mentioned on the CoA in the standard vial. Use the standard vial as Tube 1 in Figure 1. Prepare each NGAL standard in polypropylene tubes by serial dilution of the reconstituted standard with dilution buffer as shown in Figure 1*. After reconstitution the standard must be used within 15 minutes and cannot be stored for repeated use.
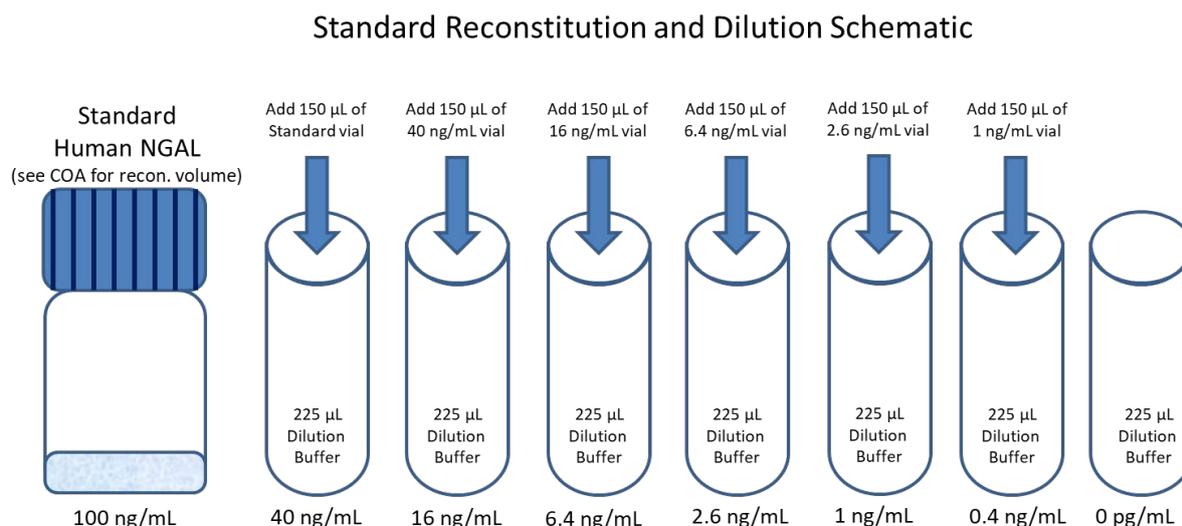
Standard Reconstitution and Dilution Schematic

| | Add 150 µL of Standard vial | Add 150 µL of 40 ng/mL vial | Add 150 µL of 16 ng/mL vial | Add 150 µL of 6.4 ng/mL vial | Add 150 µL of 2.6 ng/mL vial | Add 150 µL of 1 ng/mL vial | |

Standard Human NGAL (see COA for recon. volume)

| 225 µL Dilution Buffer | 225 µL Dilution Buffer | 225 µL Dilution Buffer | 225 µL Dilution Buffer | 225 µL Dilution Buffer | 225 µL Dilution Buffer | 225 µL Dilution Buffer |

| 100 ng/mL | 40 ng/mL | 16 ng/mL | 6.4 ng/mL | 2.6 ng/mL | 1 ng/mL | 0.4 ng/mL | 0 pg/mL |

Figure 1*) COA: Certificate of Analysis

Tracer solution

The tracer is reconstituted by pipetting 1 ml distilled or de-ionized water. Dilute the reconstituted 1 ml tracer with 11 ml dilution buffer, which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of tracer by diluting 1 part of the reconstituted tracer with 11 parts of dilution buffer.

Streptavidin-peroxidase solution

It is advised to spin down streptavidin-peroxidase tubes before use. Prepare the streptavidin-peroxidase solution by mixing 0.25 ml of the 100x streptavidin-peroxidase solution with 24.75 ml dilution buffer, which is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of streptavidin-peroxidase solution by diluting 1 part of the 100x streptavidin-peroxidase solution with 99 parts of dilution buffer.

9.   ELISA PROTOCOL

Bring all reagents to room temperature (20 - 25°C) before use.

1. Determine the number of test wells required, put the necessary microwell strips into the supplied frame, and fill out the data collection sheet. Return the unused strips to the storage bag with desiccant, seal and store at 2 - 8°C.

2. Transfer 100 µl in duplicate of standard, samples, or controls into appropriate wells. Do not touch the side or bottom of the wells.

3. Cover the tray and tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.

4. Incubate the strips or plate for 1 hour at room temperature.

5. Wash the plates 4 times with wash buffer using a plate washer or as follows*:

    a. Carefully remove cover, avoid splashing.

    b. Empty the plate by inverting plate and shaking contents out over the sink, keep inverted and tap dry on a thick layer of tissues.

    c. Add 200 µl of 1x wash buffer to each well, wait 20 seconds, empty the plate as described in 5b.

    d. Repeat the washing procedure 5b/5c three times.

    e. Empty the plate and gently tap on thick layer of tissues.

6. Add 100 µl of diluted tracer to each well using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.

7. Cover the tray and incubate the tray for 1 hour at room temperature.

8. Repeat the wash procedure described in step 5.

9. Add 100 µl of diluted streptavidin-peroxidase to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.

10. Cover the tray and incubate the tray for 1 hour at room temperature.

11. Repeat the wash procedure described in step 5.

12. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.

13. Cover the tray and incubate the tray for 30 minutes at room temperature. It is advised to control the reaction on the plate regularly. In case of strong development the TMB reaction can be stopped sooner. Avoid exposing the microwell strips to direct sunlight. Covering the plate with aluminium foil is recommended.

14. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 12. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any air bubbles trapped in the wells.

15. Read the plate within 30 minutes after addition of stop solution at 450 nm using a plate reader, following the instructions provided by the instrument's manufacturer.

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*)   In case plate washer is used, please note: use of a plate washer can result in higher background and decrease in sensitivity. We advise validation of the plate washer with the manual procedure. Make sure the plate washer is used as specified for the manual method.

10.   INTERPRETATION OF RESULTS

- Calculate the mean absorbance for each set of duplicate standards, control and samples.
- If individual absorbance values differ by more than 15% from the corresponding mean value, the result is considered suspect and the sample should be retested.
- The mean absorbance of the zero standard should be less than 0.3.
- Create a standard curve using computer software capable of generating a good curve fit. The mean absorbance for each standard concentration is plotted on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis (logarithmic scale).
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
- Samples that give a mean absorbance above the absorbance for the highest standard concentration are out of range of the assay. These samples should be retested at a higher dilution.

11.   TECHNICAL HINTS

- User should be trained and familiar with ELISA assays and test procedure.
- If you are not familiar with the ELISA technique it is recommended to perform a pilot assay prior to evaluation of your samples. Perform the assay with a standard curve only following the instructions.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing wash buffer, fill with wash buffer as indicated for each cycle and do not allow wells to sit uncovered or dry for extended periods.
- Since exact conditions may vary from assay to assay, a standard curve must be established for every run. Samples should be referred to the standard curve prepared on the same plate.
- Do not mix reagents from different batches, or other reagents and strips. Remainders should not be mixed with contents of freshly opened vials.
- Each time the kit is used, fresh dilutions of standard, sample, tracer, streptavidin-peroxidase and buffers should be made.
- Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
- To avoid cross-contaminations, change pipette tips between reagent additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- The waste disposal should be performed according to your laboratory regulations.

Technical support

Do not hesitate to contact our technical support team at techsupport@diapharma.com for inquiries and technical support regarding the human NGAL ELISA.

12. QUALITY CONTROL

The Certificate of Analysis included in this kit is lot specific and is to be used to verify results obtained by your laboratory. The absorption values provided on the Certificate of Analysis are to be used as a guideline only. The results obtained by your laboratory may differ.

This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Hbt immunoassay, the possibility of interference cannot be excluded.

For optimal performance of this kit, it is advised to work according to good laboratory practice.

13. TROUBLESHOOTING

Suggestions summarized below in Table 2 can be used as guideline in case of unexpected assay results.

Low absorbance	High absorbance	Poor duplicates	All wells positive	All wells negative	Possible cause
•	•		•	•	Kit materials or reagents are contaminated or expired
•					Incorrect reagents used
•		•	•		Lyophilized reagents are not properly reconstituted
•	•	•	•	•	Incorrect dilutions or pipetting errors
•		•			Improper plastics used for preparation of standard and/or samples
•	•				Improper incubation times or temperature
		•			Especially in case of 37°C incubation: plates are not incubated uniformly
•					Assay performed before reagents were adapted to room temperature
•	•	•	•	•	Procedure not followed correctly
				•	Omission of a reagent or a step
		•			Poor mixing of samples
	•		•		Low purity of water
	•	•			Strips were kept dry for too long during/after washing
	•	•	•		Inefficient washing
	•	•			Cross-contamination from other samples or positive control
		•	•		TMB solution is not clear or colorless
•	•				Wrong filter in the microtiter reader
	•	•			Airbubbles
		•			Imprecise sealing of the plate after use
•					Wrong storage conditions
•					Lamp in microplate reader is not functioning optimally

Table 2

14. REFERENCES

1. Szewczyk, M et al; Plasma Neutrophil Gelatinase-Associated Lipocalin (NGAL) Correlations With Cystatin C, Serum Creatinine, and Glomerular Filtration Rate in Patients After Heart and Lung Transplantation. Transplantation Proceedings 2009*, 41:*3242