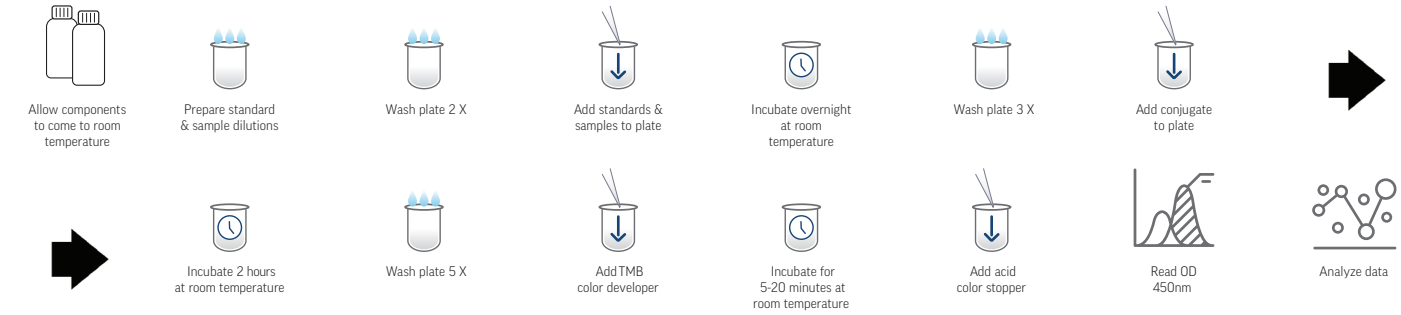
RAT NGAL ELISA



DESCRIPTION

A direct antigen capture antibody sandwich assay for the quantitative determination of rat NGAL.

Intended Use: Ethos Biosciences Rat NGAL ELISA is an immunoassay for the quantitative determination of rat NGAL in rat specimens. The assay is intended for research use; it is not intended for diagnostic purposes.

Technical Background: Rat NGAL ELISA is an antigen capture antibody sandwich ELISA run in direct mode. It uses a recombinant rat NGAL for standard and relies on two different antibodies raised against rat NGAL. These antibodies show no cross-reactivity with either rat immunoglobulins or rat albumin.

The rat NGAL ELISA Plate is supplied dry, but is ready to use. It is coated with anti-rat NGAL Ab. In practice, the plate is first washed and then controls, standard dilutions and (diluted) samples are added.

During an overnight incubation, rat NGAL is captured by the immobilized antibody. Washing the plate removes unreacted materials, and a second anti-rat NGAL antibody conjugated with horseradish peroxidase (HRP) is added. Over a two-hour incubation period this antibody-conjugate binds with rat NGAL bound to the plate.

Unreacted conjugate is removed by washing the plate, and bound conjugate is detected using a chromogenic substrate for HRP. After a short color development period, acid stopper is added, and the absorbance is measured using a plate reader.

The absorbance (determined at 450 nm) is directly proportional to the concentration of rat NGAL in the standards and samples. Dose-Responses for standard dilutions of recombinant rat NGAL, and those for serially-diluted rat urine are colinear or parallel when plotted appropriately. This indicates that the standard and antibodies are appropriate for measuring rat NGAL in rat samples.

The standard curve for this assay runs from 0.31-20 ng rat NGAL/mL.

Kit Contents:

- 1 x 96-well Rat NGAL Assay Plate
- 2 x NHE-Casein Diluent
- 1 x Rat NGAL Standard
- 1 x Anti-Rat NGAL-HRP Conjugate
- 1 x TMB Color Developer
- 1 x Acid Color Stopper
- 1 x Instructions

All kit reagents are supplied in ready to use liquid form.

Note: Items 2 through 4 contain 0.05% Proclin® 300 (active component Isothiazolinone) as preservative.

Color Stopper contains 2.0 N Sulfuric Acid. Refer to the SDS online at Ethos Biosciences' website for details. Save and store all unused reagents and strips (in sealed pouch with included desiccant pack) for future use at 4ºC. Do not use this kit beyond its expiration date.

REAGENTS AND EQUIPMENT (NOT SUPPLIED) REQUIRED TO COMPLETE THE ASSAY:

1. EIA Wash Buffer: 0.15M NaCl, 0.01M Triethanolamine, pH 6.8, 0.05% Tween® 20 prepared in deionized water. A preservative, i.e., 0.05% v/v Proclin® 300 may be added to prevent microbial growth. Proclin 300 will not adversely affect either antibody binding or HRP enzymatic activity. **Azide is unsuitable for use as preservative for this wash buffer.**
2. Plate washing equipment: This may be a simple wash bottle or an automated plate washer.
3. Incubation container: ELISAs are sensitive to temperature, and edge effects have been found with plates left open during incubations. These problems can be minimized by completing all incubations in a tightly-closed plastic container. A water moistened paper towel (on the bottom of the plastic container) serves to control humidity.
4. Micropipettors (and tips) capable of delivering volumes from 1-1000 µL.
5. Multichannel pipettor that can deliver 100 µL volumes is recommended
6. Tubes for dilution of standard and samples: 1.5 mL microfuge tubes work well in this capacity.
7. Plate Reader: The plate reader should be capable of measuring absorbance at 450 nm.

SPECIMEN COLLECTION AND STORAGE:

Samples should be clear and free of hemolysis.

Rat urine: Collect urine samples without preservative. DO NOT CENTRIFUGE. Vortex the sample then freeze. Urine samples may be stored frozen at -70˚C for up to 2 months. *Avoid repeated freeze-thaw cycles.*
Rat serum: Allow clotting of rat blood for at least 30 minutes. Centrifuge for 10 minutes at 1000 x g. Recover serum layer. Assay immediately or store serum samples at -70°C.
Avoid repeated freeze-thaw cycles.
Cell Culture Supernatant/ Cell Lysate: Centrifuge all samples to remove debris prior to analysis. Assay immediately or store samples at -70°C. *Avoid repeated freeze-thaw cycles.*

Limitations:
It is the responsibility of the investigator to determine if the presence of experimental compounds or their metabolites in the urine may affect the assay results.

Gross hemolysis of the sample may affect assay results.

Gross microbiological contamination may affect assay results.
Samples should be run at multiple dilutions to determine the optimal dilution factor for accurate quantitation.

ASSAY PROCEDURE:

Prepare wash buffer and set-up equipment to wash the plate(s). At least one liter of wash buffer should be prepared.

Washing can be completed using a squirt bottle at the sink, and a method that has proven effective is described below.
Allow all reagents and materials to come to room temperature. The assay performs well when conducted at 20-25° C.

Standard and Samples, once at room temperature, should be gently mixed then allow any sediment to settle for 30 - 60 minutes. Alternatively, centrifuge the sample at 1000 x g for 1 minute. Use the supernatant in the assay.

These instructions are written with the intent to complete the assay using duplicate wells for each dilution of standard, and each dilution of sample. The Rat NGAL ELISA may be used to determine the concentration of up to 40 samples if performed at a single dilution.

Standard Dilutions: The standard is supplied as a two-fold concentrate. This procedure describes the preparation of seven (7) two-fold serial dilution of standard. Standards should be prepared fresh.

1. Prepare 7 microfuge tubes with 225 µL of NHE-Casein Diluent per tube.
2. Label the tubes numbers 1-7.
3. Transfer 225 µL of Rat NGAL Standard Stock to tube 1; this is a 1:2 dilution of the standard.
4. Mix contents by aspirating and expelling the fluids 5 times.
5. Transfer 225 µL of solution from tube 1 to tube 2.
6. Mix as before.
7. Continue this procedure through tube number 7.
8. Tubes 1-7 now contain dilutions representing 20, 10, 5.0, 2.5, 1.25, 0.63, 0.31 ng/ml rat NGAL. Undilute standard (40 ng/ml) is possible if needed to extend the standard curve.

Preparation of Sample Dilutions: Samples must be diluted in NHE-Casein to fall within the range of the assay:
- The user needs to estimate the concentration of the NGAL in the sample and select the correct dilution factor.
- A starting dilution of 1:5 for rat urine specimens is suggested. More concentrated samples may show urine effects but may be possible. For initial studies, and particularly if both normal and diseased samples are represented, it is wise to complete the analysis at more than one dilution, for example 1:5, 1:10, 1:20.
- A starting dilution of 1:50 for rat serum specimens is suggested. Again, for initial studies, particularly when normal and diseased samples are represented, it is wise to complete the analysis at more than one dilution.

It is recommended that sample dilutions be performed in tubes; dilution in the plate is not recommended.

Addition of Controls, Standard NGAL Dilutions and Samples to the plate:
- Prepare a record for the assay taking care to include a plate map.
- Assemble diluent standard dilutions and diluted samples.
- Remove the plate from packing, and label the strips of the plate with an indelible marker 1-12. This will allow reconstruction of the plate should strips fall out during the washing procedures.

Wash the plate for 2 cycles: using a squirt bottle, multichannel pipet or

automatic plate washer. One cycle consists of:

1. Aspirating fluid from the plate if necessary (flipping out wash buffer into the sink works well).
2. Filling each well with EIA Wash Buffer
3. Aspirating each well in the plate; Complete removal of liquid after each cycle is essential for good performance.

After the last cycle invert the plate on a clean paper towel and tap gently to blot any adherent fluids. Do not allow plate to dry out.
The plate design described here includes a negative control termed C0. This placed in wells A1, and A2. All other wells receive either diluted standard or diluted sample. The standard and/or sample assay volume is 100 µL per well.

Primary Incubation: Reaction with capture anti-Rat NGAL antibody on the plate.

1. Add 100 µL NHE-Casein Diluent from the stock bottle to wells A1 and A2. This is the negative control "C0" and will be used to standardize or "blank" the microplate reader.
2. With a fresh tip, transfer 100 µL aliquots of NGAL Standard Dilution Tube 1 (1:2 dilution of stock) to wells B1 and B2.
3. With a fresh tip, transfer 100 µL aliquots NGAL Standard Dilution Tube 2, and to wells C1 and C2.
4. Continue transferring diluted standard to the plate in this fashion, i.e., in order through H1 and H2, taking care to use a fresh tip for each new dilution.
5. Using a new tip, add 100 µL aliquots of diluted sample to wells A3 and A4.
6. Continue adding diluted samples to the plate, taking care to change the tip for each one.
7. Place a water moistened paper towel in the bottom of the incubation "chamber," place the plate on top, and cover the container.
8. Incubate at room temperature (RT) overnight.

Wash the plate for 3 cycles, as described above

Secondary Antibody Incubation: Reaction with detector anti-Rat NGAL-HRP antibody Conjugate:

1. Add 100 µL of anti-rat NGAL-HRP Conjugate to every well on the plate. Use of a multichannel pipettor set to deliver 100 µL is recommended.
2. Cover and incubate the plate for 2 hours at room temperature in a moist chamber.

Wash the plate for 5 cycles, as described

Color Development:
1. Add 100 µL of Color Developer to each well. The wells will turn blue with the intensity directly proportional to the concentration of NGAL in the well.
2. Develop 5- 20 minutes.
3. Add 100 µL of Color Stopper to each well. The color will change from blue to yellow.

Measure Absorbance: Use a plate reader to determine and record the absorbance of all experimental wells at 450 nm, blanked against well A1. If the reader is capable of reading at 630 nm, the absorbance reading at 630 nm may be subtracted from the 450 nm reading.

Analysis:
The results are analyzed using a computer-based curve-fitting software program for a 4 or 5 parameter logistics (PL) curve fitting algorithm plotting the mean absorbance (y axis) against the protein concentration (x axis). The NGAL concentration of the samples can then be interpolated from the standard curve. Multiply the concentration by the dilution factor to determine the concentration of the undilute sample.

Alternatively, if a 4 PL fit program is not available, prepare a spreadsheet entering appropriate data including standard dilution, concentration, sample dilution and absorbance data. Determine the mean for replicate wells.

Prepare a plot of standard dilutions with the NGAL concentration (ng/ml) on the x-axis and the mean absorbance on the y-axis.

The data that fall into the linear portion of the dose-response curve constitute the usable portion of the assay.

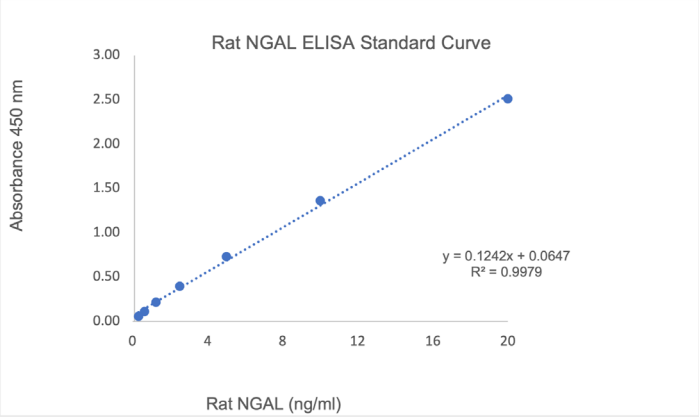
Subject these data to linear analysis to yield a mathematical model, of the form:

$$Abs = m\,[NGAL] + b$$

which rearranges into:

$$[NGAL] = (A_{450} - b) / m$$

NGAL concentration of an undilute sample is determined by taking the calculated values from this equation and multiplying by the dilution factor.



Quality Control:
- Record Keeping: It is good laboratory practice to record the lot numbers and dates of the kit components and reagents for each assay.
- Sample Handling: The samples should be secured, processed and stored as discussed above.
- Dilute Standard and Samples carefully. For each standard and sample, a fresh tip should be used.
- Template: Record the position of each standard or sample on a microplate template.

Trouble Shooting: Reaction with capture anti-Rat NGAL antibody on the plate.

1. No color appears after adding Color Developer: One or more reagents may not have been added. Repeat assay. Be sure to store the kit appropriately. Do not use expired kits or reagents from other kits
2. Color in wells too light: Longer incubation with Color Developer may be required. If the color is still too light after 20 minutes development, repeat the assay but increase the secondary incubation to 4 hours.
3. Color in wells is too dark: Be sure to wash with the appropriate buffer. Be sure to blot dry after washing. Do not allow wells to dry out. Ensure that the standards and samples were added to the plate first followed by the primary antibody. Decrease the development time. If a 5-minute development yields the C1 too dark with no appropriate dose-response of the standard curve, repeat the assay and reduce the secondary incubation to 1 hour. If color is dark and the standard dilutions fail to show the appropriate dose-response, Color Developer may have been contaminated with conjugate or the plate was poorly washed. The color developer should be clear to a very pale blue at room temperature. Be sure that all reagents, standards and samples are at room temperature throughout the assay. Repeat the assay and take care in the pipetting and in the washing operations.
4. Color in sample well(s) is lighter or darker than lowest or highest concentrations of the standard curve. Change sample dilution appropriately.
5. Poor agreement between duplicate wells: This is almost always due to pipetting error. Repeat the assay.
6. Microplate ELISAs may be prone to edge effects (or prozone effect) wherein the outer rows and columns show a darker response than the inner ones. This effect may be minimized by incubating the plate in a closed humid container. A plastic container with a tight-fitting lid and containing a water-moistened paper towel works well in this respect. Place the moistened towel in the bottom of the container and place the microplate upon it; add enough room temperature water to barely touch the bottom of the wells. Position the cover and incubate as described. When removing the plate, be sure to gently blot the bottom of the wells to remove excess water before continuing with the wash cycles and assay steps.

PRODUCT INFORMATION

Cat. #	Description
1039	Rat NGAL ELISA

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