ab207925
Rat Lipocalin-2 ELISA Kit

Instructions for use:

For the *in vitro* determination of Rat NGAL in urine, plasma or serum, tissue extracts or culture media.

This product is for research use only and is not intended for diagnostic use.

Version 5 Last Updated 15 January 2019

Table of Contents

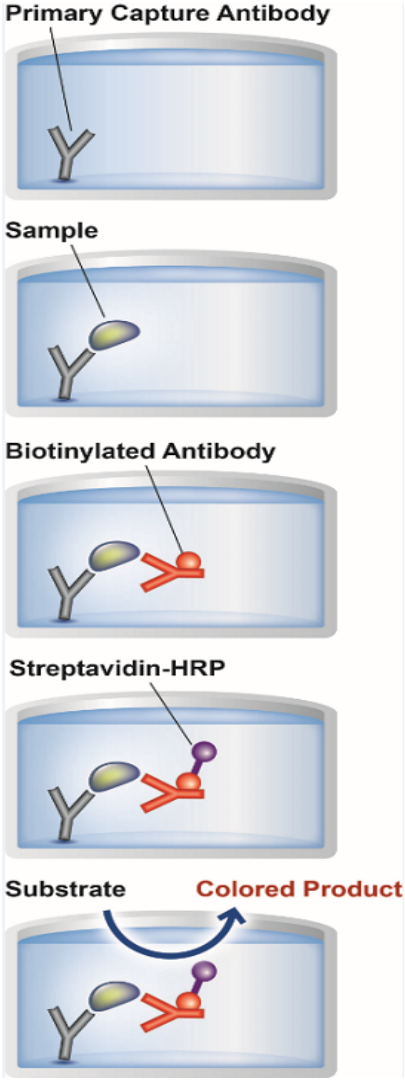
1. BACKGROUND

Abcam's Rat Lipocalin-2 ELISA Kit (ab207925) is an *in vitro* enzyme-linked immunosorbent assay (ELISA) for the quantitative measurement of neutrophil gelatinase-associated lipocalin levels in urine, plasma or serum, tissue extracts or culture media samples.

The assay is a sandwich ELISA performed in microwells coated with a mouse monoclonal antibody against Rat 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.

NGAL (neutrophil gelatinase-associated lipocalin) belongs to the lipocalin family of proteins. These are secreted proteins characterized by their ability to bind small hydrophobic molecules in a structurally conserved pocket formed by β-pleated sheet, to bind to specific cell-surface receptors, and 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. In the rat, NGAL was first named 25 kDa α_2-microglobulin-related protein, and later *neu*related lipocalin (NRL) 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, 24-kDa superinducible protein (SIP24) or uterocalin.

2. ASSAY SUMMARY



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

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.

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.

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.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as hazardous and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. STORAGE AND STABILITY

Store kit at 4ºC upon receipt. Do not freeze. The kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

5. LIMITATIONS

- This ELISA kit is intended for research use only. It is not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Microwell plate, 96 pre-coated wells	96 Wells	4ºC	RT
5x Sample Diluent Buffer	50 mL	4ºC	RT
Rat NGAL Standard (0 pg/mL)	1 mL	4ºC	RT
Rat NGAL Standard (4 pg/mL)	1 mL	4ºC	RT
Rat NGAL Standard (10 pg/mL)	1 mL	4ºC	RT
Rat NGAL Standard (20 pg/mL)	1 mL	4ºC	RT
Rat NGAL Standard (40 pg/mL)	1 mL	4ºC	RT
Rat NGAL Standard (100 pg/mL)	1 mL	4ºC	RT
Rat NGAL Standard (200 pg/mL)	1 mL	4ºC	RT
Rat NGAL Standard (400 pg/mL)	1 mL	4ºC	RT
25x Wash Buffer Solution	40 mL	4ºC	RT
Biotinylated Rat-NGAL Antibody	12 mL	4ºC	RT
HRP-Streptavidin Solution	12 mL	4ºC	RT
TMB Substrate Solution	12 mL	4ºC	RT
Stop Solution	12 mL	4ºC	RT

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Adjustable micropipettes covering the range 1-1000 µL and corresponding disposable pipette tips.
- Polypropylene tubes to contain up to 2000 µL.
- Tube racks.
- Adjustable 8 or 12 channel micropipette (50 - 250 µL range) or repeating micropipette (optional).
- Clean 1 L and 250 mL graduated cylinders.
- Deionized or distilled water.
- Cover for microplate.
- Clean container for diluted Wash Solution.
- Microplate washer for wash steps (optional).
- Lint-free paper towels or absorbent paper.
- Disposable pipetting reservoirs.
- Timer (60-minute range.)
- Calibrated ELISA plate reader capable of reading at 450 nm (preferably subtracting reference values at 650 or 620 nm).
- Sodium hypochlorite (household bleach 1:10 dilution) for decontamination

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' refers to a single assay well. Review the protocol completely to confirm this kit meets your requirements. Please contact our Scientific Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay
- Samples which generate values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Completely aspirate all solutions and buffers during wash steps.

9. REAGENT PREPARATION

- 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).

9.1. **Wash Buffer Solution:**

Dilute the 25x Wash Buffer Solution To make a 1x Wash Buffer Solution 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 4°C after use.

9.2. **Sample Diluent Buffer:**

Dilute the 5x Sample Diluent Buffer to make the 1x Sample Diluent Buffer 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 4°C after use.

9.3. **Biotinylated Rat-NGAL Antibody (ready to use):**

Do not dilute further.

9.4. **HRP-Streptavidin Solution (ready to use):**

Do not dilute further.

9.5. **TMB Substrate Solution (ready to use):**

Do not dilute further.

9.6. **Stop Solution (ready to use):**

Do not dilute further.

10. STANDARD PREPARATION

10.1. **Rat NGAL Standards 0 to 400 pg/mL (ready to use):**

Do not dilute further. The assigned concentration of each standard is indicated on its label.

11. SAMPLE PREPARATION

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1. General Sample Information

11.1.1. Handle and dispose of all blood-derived or urine samples as if they were potentially infectious.

11.1.2. Determination of NGAL in a single sample requires 10 µL 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 analysed 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.

11.2. **Samples:**

11.2.1. Dilute each sample in a recorded proportion with the 1x Sample Diluent Buffer to obtain at least 250 µL of diluted solution that can be set up in duplicate wells at 100 µL per well.

11.2.2. 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 990µL of 1x Sample Diluent Buffer to make a 1/100 dilution; then dilute 10 µL of the 1/100 dilution in 1990 µL of 1x Sample Diluent buffer to make a 1/20,000 dilution.

11.2.3. Dilutions are mixed by inversion or gentle vortexing.

Samples should be re-assayed at higher or lower dilution as appropriate if the measured absorbance is outside of the concentrations of the standard curve.

12. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **We recommended to assay all standards, controls and samples in duplicate.**
- **Prepare all reagents, including 1x buffers, and samples as directed in the previous sections.**

12.1. Prepare the assay protocol, assigning the appropriate wells for setting up standard protein, 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 1x Sample Diluent Buffer instead of diluted sample and processed like the other wells.

12.2. Pipette 100 µL volumes of each standard solution (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 rpm.

12.3. Aspirate the contents of the microwells and wash them three times with 300 µL 1x Wash Buffer Solution. If the washing is done manually, empty the microwells by inversion, 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.

12.4. Dispense 100 µL of Biotinylated Rat NGAL antibody (ready to use) into each microwell. Cover the wells and incubate for 60 minutes at room temperature on a shaking platform set at 200 rpm.

12.5. Wash as described above in Step 12.3.

12.6. Dispense 100 µL of HRP-Streptavidin Solution (ready to use) into each microwell. Cover the microplate and incubate for 60 minutes at room temperature on a shaking platform set at 200 rpm.

12.7. Wash as described above in Step 12.3.

12.8. Dispense 100 µL of TMB Substrate Solution (ready to use) into each microwell. Start the clock when filling the first well. Cover the wells and incubate for exactly 10 minutes at room temperature in the dark.

12.9. Add 100 µL Stop Solution (ready to use) to each well, maintaining the same pipetting sequence and rate as in Step 12.8. Mix by gentle shaking for 20 seconds, and avoid splashing. Measure the microplate within 30 minutes of adding the Stop Solution.

12.10. Measure the absorbance of the microplate at 450 nm in a microplate reader using a reference wavelength 650 or 620 nm. If no reference wavelength is available, is subtract the value of the blank well from each of the absorbance of the other wells before additional calculations are performed.

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard solution should be further diluted in appropriate buffer and re-analyzed, then multiply the concentration found by the appropriate dilution factor.
- Results can 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 a linear or 4-parameter curve fit.
- Diluted samples that give a mean absorbance above that for the Rat NGAL standard 8 or below that for the Rat NGAL standard 2 are out of the range of the assay and their concentrations should be noted as >400 pg/mL and <4 pg/mL, respectively.

13.1. **Validation of calibration curve:** The mean absorbance value for the 400 pg/mL Rat NGAL standard should be >1.5. The mean absorbance value for any Rat NGAL standards should be higher than that for the next lower standard, e.g. standard 7 (200 pg/mL) should give a higher reading than standard 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 standards: One or more individual standard may give anomalous absorbance readings. This error is significant if it impairs satisfactory curve fitting by the 4-parameter logistic method, which, as a result of the outlier value, is shifted away from other standard absorbance values that are in fact correct. The standard points and fitted curve should always be examined for correct before any calculations of concentration from it are accepted. A poorly fitting curve will also be revealed by a high sum of residual squares.

13.2. If only one standard value is affected, which is not the highest standard, two courses of action are possible:

13.2.1. An erroneous single 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.

13.2.2. 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 outlier.

NOTE**:** *If two or more standards are affected, the assay should be repeated.*

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed

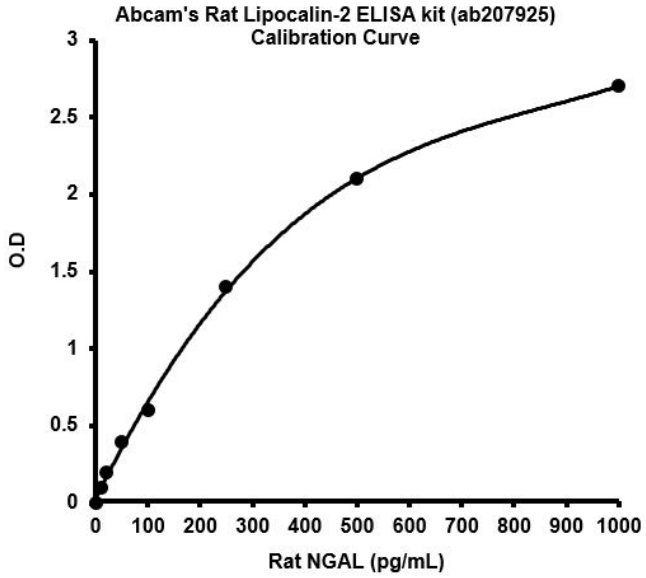


Figure 1 Abcam's Rat Lipocalin-2 ELISA Kit (ab207925) Calibration Curve

- **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 to 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 strains of Rat.
- 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.

- **Performance characteristics**: **Limit of detection**: The lowest concentration of rat NGAL giving an absorbance reading greater than 2 standard deviations above the mean zero standard reading (n = 20) is 0.5 pg/mL.
- **Precision: Intra-assay** 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):

Sample	CV (%)
Urine 1	3
Urine 2	3
Plasma 1	3
Plasma 2	4

- **Inter-assay** 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:

Sample	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 Standard material and analyzed in the assay. Recovery was calculated from (measured value)/(expected value) expressed as a percentage.

Sample	Measured (pg/mL)	Expected (pg/mL)	Recovery (%)
U1	61	55	111
U2	101	93	109
U3	183	179	102
U4	350	346	101
P1	54	50	108
P2	92	88	105
P3	173	174	99
P4	349	341	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.

15. QUICK ASSAY PROCEDURE

NOTE: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

15.1. Assign the appropriate wells for setting up standards, diluted samples and any internal laboratory controls in duplicate.

15.2. Pipette 100 µL of standard dilution, diluted samples and internal controls into their corresponding wells. Cover and incubate for 60 minutes at room temperature on a shaking platform set at 200 rpm.

15.3. Aspirate the contents of the wells and wash them three times with 300 µL 1x Wash Buffer Solution.

15.4. Dispense 100 µL of Biotinylated Rat-NGAL antibody into each well. Cover the wells and incubate for 60 minutes at room temperature on a shaking platform set at 200 rpm.

15.5. Wash as described above.

15.6. Dispense 100 µL of HRP-Streptavidin solution into each well. Cover the wells and incubate for 60 minutes at room temperature on a shaking platform set at 200 rpm.

15.7. Wash as described above.

15.8. Dispense 100 µL of TMB Substrate solution into each well. Start the clock when filling the first well. Cover and incubate for exactly 10 minutes at room temperature in the dark.

15.9. Add 100 µL Stop Solution to each well. Mix by gentle shaking for 20 seconds, avoid splashing. Read the wells within 30 minutes.

15.10. Read the absorbance values of the wells at 450 nm in a microplate reader (reference wavelength 650 or 620 nm).

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Clear plates
Sample with erratic readings	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances.
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol
Standard readings do not follow a linear	Pipetting errors in standard or reaction	Avoid pipetting small volumes (< 5 µL)

Problem	Cause	Solution
pattern	mix	and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

18. NOTES

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