

# **biosensis<sup>®</sup> Rat Lipocalin-2/NGAL ELISA Kit Protocol**

**Catalogue No: BEK-2128-1P**

*For quantitative detection of rat NGAL/Lipocalin-2 in cell culture supernatants, urine, serum and heparin treated plasma samples when used as directed.*

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## I. Materials Provided

<b>Kit Components</b>	<b>Unit Size/Volume</b>
Lyophilized recombinant Rat NGAL standard	10ng/tube x 2
96 wells plate pre-coated with anti- Rat anti-NGAL antibody	1 x plate
Sample diluent buffer	30ml x 1
Biotinylated anti- rat NGAL (dilution 1:100)	130µl x1
Antibody diluent buffer	12ml x 1
Avidin-Biotin-Peroxidase Complex (ABC) (dilution 1:100)	130µl x 1
ABC diluent buffer	12ml x 1
TMB colour developing agent	10ml x 1
TMB stop solution	10ml x 1
PBS Wash Buffer Concentrate Packet	1 pk x 1
Plate Sealers	1 ea x 4

## II. Equipment Required but Not Supplied

1. Distilled Water, 1000 mL +, graduated cylinders for measuring
2. Automated plate washer is desirable
3. Multi-channel or repeating pipette.
4. Clean tubes and Eppendorf tubes.
5. Plate shaker (300-500 rpm)
6. Mechanical Vortex.
7. Microplate reader with 450nm filter.

## III. Technical Hints

1. Spin all kit components to get liquid to the bottom of tubes before use.
2. Duplicate well assays are recommended for both standard and sample testing.
3. In order to avoid marginal effects of plate incubation due to temperature differences, it is suggested that the ABC and TMB solutions be brought to room temperature 30 min before use.
4. Recommended method for manual plate washing.
  - Emptying of wells on the plate:

Place the plate on the palm of the hand in a position that enables easy flicking movement using the wrist. Holding the plate over a sink, quickly invert the plate,

whilst accelerating the arm downward toward the sink. Abruptly stop the downward acceleration to force the liquid from the wells into the sink. When done correctly the technique should prevent liquid from getting on to the fingers or on the outside of the strip wells or plate holder. *Note: Retain the upside down position of the plate to avoid any back flow into the wells.*

- Blotting the plate:  
Immediately blot the inverted plate by lightly tapping the plate 3-4 times on blotting paper.
- Washing: Forcefully pipette Wash Buffer into each well with a multi-channel pipette. Empty the wells of wash buffer using technique described above. Repeat washing and flicking procedures thrice. Wash plate with 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 min before rinsing with more wash buffer as directed above. *Note: Avoid touching the inside surface of the wells with the pipette tips.*

***Do not let the wells dry out at any time or enzymatic activity will be lost.***

#### 5. Preparation of Wash buffer:

Preparation of 0.01 M PBS: Dissolved enclosed packet contents in 900ml distilled water and adjust pH to 7.2-7.6, finally adjust to 1000 mL. Store at 4°C 1-2 weeks, warm to room temperature before use. Use sterile technique, buffer contains no preservatives. Do not reuse.

*Note: if blank values are over 0.2 the addition of 0.05% Tween-20 PBS wash buffers will usually help lower them as well as overall assay backgrounds and can help improve signal/noise ratios.*

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## IV. Storage of Kit Components

This kit may be stored for up to 6 months at 2°C to 8°C from the date of shipment. Standards should be stored at -20°C or -80°C (recommended at -80°C) after reconstitution. Coated strips or reagents may be store for up to 1 month at 2°C to 8°C. Return unused wells to the pack and reseal pack. *Note: the kit can be used within 8 months if the whole kit is stored at -20°C. Avoid repeated freeze-thaw cycles.*

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## V. Sample Preparation and Dilution

- **Cell culture supernatant:** Remove particulates by centrifugation (10,000 x g 5 min), analyze immediately or aliquot and store at -20°C-80°C. Divide samples into aliquots to prevent multiple freeze thaws; use appropriate protease inhibitor cocktails for best results.
- **Urine:** Aseptically collect. Remove particular impurities and precipitates by centrifugation, and/or particle removal via syringe or another filtration device. Solution must be particle and cell free for best results. Assay immediately or aliquot and store samples at -20°C to -80°C. Note: treatment of urine with Thimerosal or other proteinase inhibitors can increase signal yields in ELISA assays for some targets, please see: (Hoyer, J. et al. (1999). Effects of microgravity on urinary osteopontin. J. Am. Soc. Nephrol. 10: S389-S393.) Note 2: Urine can be quite acidic; neutralize with mild base the final assay sample to pH 7.0-7.5 for best results.
- **Serum:** Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 1000 X g for 15 min. Analyze the serum immediately or aliquot and store frozen at -20°C-80°C.
- **Plasma:** Collect plasma using heparin only as an anticoagulant. Centrifuge for 15 min at 2-8°C at 1000 x g within 30 min of collection. For eliminating the platelet effect, suggesting that further centrifugation for 10 min at 2-8°C at 10000 x g. Analyze immediately or aliquot and store samples at -20°C-80°C.

*Note: The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard curve.*

The following table can be used as a guideline for sample dilution. Dilute the sample using the provided diluent buffer. Mix the sample thoroughly with diluent buffer.

Target protein concentration	Concentration range	Working dilution	Amount of sample diluent buffer to be added
<b>High target protein concentration</b>	<b>50-500 ng/ml</b>	1:100	Add 3 µl sample into 297 µl sample diluent buffer
<b>Medium target protein concentration</b>	<b>5-50 ng/ml</b>	1:10	Add 25 µl sample into 225 µl sample diluent buffer
<b>Low target protein concentration</b>	<b>78.125 pg/ml-5000 pg/ml</b>	1:2	Add 100 µl sample to 100 µl sample diluent buffer
<b>Very Low target protein concentration</b>	<b>≤78.125 pg/ml</b>	No dilution necessary	(working dilution is 1:2)

Sensitivity <10pg/ml

\*The sensitivity or the minimum detectable dose (MDD) is the lower limit of target protein that can be detected by the kit. It is determined by adding two standard deviations to the mean O.D. value of twenty (20) blank wells and calculating the corresponding concentration .Range: 78pg/ml-5000pg/ml; Specificity: Natural and recombinant Rat Lcn2 There is no detectable cross-reactivity with other relevant proteins

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
N	16	16	16	16	16	16
Mean (pg/ml)	121	750	3168	126	727	3167
Std. Dev.	6.05	50.25	168.96	7.3	56.7	177.35
CV(%)	5.0	6.7	5.3	5.8	7.8	5.6

## VI. Reagent Preparation

### 1. Preparation of Standards

- Reconstitution of standard with the sample diluent provided.
  - (a) 10,000pg/ml of standard solution: It is recommended that the standards be prepared no more than 2 hours prior to performing the experiment. Use one 10ng of lyophilized Rat Lcn2 standard for each experiment. Gently spin the vial prior to use. Reconstitute the standard to a stock concentration of 10ng/ml using 1ml of sample diluent.
  - (b) To generate standard #1, add 500 $\mu$ l of the reconstituted standard stock solution of 10ng/ml and 500 $\mu$ l of sample diluent to tube #1 for a final volume of 1000 $\mu$ l. Mix thoroughly. This is tube #1 standard for the assay. Allow the standard to sit for a minimum of 10 minutes with gentle agitation prior to making dilutions. Standard is recombinant rat NGAL expressed in and purified from a mammalian cell expression system, amino acids 21-198 of rat NGAL.
  - (c) 2500pg/ml to 78 pg/ml of rat NGAL standard solutions: Number tubes 2-8. Final Concentrations to be Tube # 1 –5000pg/ml, #2 –2500pg/ml, #3 – 1250pg/ml, #4 – 625pg/ml, #5 – 312.5pg/ml, #6 –156.25pg/ml, #7 – 78.125pg/ml, #8 – Sample Diluent serves as the zero standard (0pg/ml). Aliquot 0.3 ml of sample diluent buffer into to tubes # 2-8. THEN Add 0.3 ml of the above 5000 pg/ml standard solution (tube #1 from (b), into tube 2 and mix. Transfer 0.3ml from tube 2 in to tube 3 and mix. Continue performing serial dilutions, do not add anything to tube 8 (zero tube, use only sample buffer here)
- *Note: Standard solution should be prepared no more than 2 hours prior to the experiment. The working standard solution may be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.*

### 2. Preparation of Biotinylated antibody working solution

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).
- Biotinylated antibody (Reagent B) should be diluted in 1:99 with the antibody diluent buffer and mixed thoroughly.
- *Note: the solution should be prepared no more than two hours prior to the experiment.*

### 3. Avidin-Biotin-Peroxidase Complex (ABC)

- The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume).
- ABC should be diluted in 1:99 with the ABC diluent buffer and mixed thoroughly.
- *Note: the solution should be prepared no more than one hour prior to the experiment.*

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## VII. Assay Procedure

We recommend that standard solution dilutions and each sample are plated in duplicate. Read entire protocol before beginning; if using optional method the ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use.

1. Aliquot out the standards into each well of the pre-coated plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample into each empty well. We recommend at least duplicate standards and samples. *Note: See “VII. Sample Preparation and Dilution Guideline” above for details.*
2. Seal the plate with parafilm and incubate the plate at 4°C overnight. *Note: we recommend users leave the plate on a shaker if possible.* (Optional method: 37°C, 90 minutes but it is slightly less sensitive)
3. Remove the cover, discard the plate contents and blot the plate onto paper towels or other absorbent material. **DO NOT** let the wells completely dry at any time. Wash plate 3-5 times using wash buffer as described in the technical hints. *Note: See “V. Technical Hints - Recommended method for manual plate washing” above for details.*
4. Add 0.1ml of biotinylated antibody working solution into each well. Cover and incubate the plate at room temperature on a plate shaker for 2-3 hours. (Optional 37°C, 60 minutes with shaking) Wash 3X as described in the technical hints.
5. Add 0.1ml of prepared ABC working solution (*Note: See “VIII. Reagent Preparation” above for details*) into each well. Cover and incubate the plate at room temperature on a plate shaker for 1 hour. (Optional 37°C, 30 minutes with shaking). Wash 5X as described in technical hints allowing the wash buffer to remain in the wells for 1-2 minutes.
6. Warm TMB to room temperature (37° if using optional protocol). Add 90 ul of prepared TMB color developing agent into each well and incubate **in the dark** for 30-35 minutes at RT (or 15-30 minutes at 37°C). The optimal incubation time must be empirically determined. A guideline to look for is blue shading for the top four standard wells, while the remaining standards remain clear. Stopping the reaction too quickly can lead to low standard curve values. *Note: depending upon the sample dilution the experimental samples may show color much faster, if this is the case repeating the assay at a lower sample dilution is recommended if sample O.Ds are out of range.*
7. Stop the reaction by adding 0.1ml of prepared TMB stop solution into each well. The color will change into yellow.
8. Immediately read the plate at 450nm with a microplate reader. *Note: Color will fade over time; hence, we recommend plate to be read within 30 min after adding the stop solution.*

**CAUTION:** Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.

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## VIII. Calculation of Results

### (a) Manual Plate Reading:

(The relative O.D.<sub>450</sub>) = (the O.D.<sub>450</sub> of each well) – (the O.D.<sub>450</sub> of Zero well).

The standard curve can be plotted as the relative O.D.<sub>450</sub> of each standard solution (Y) vs. the respective concentration of the standard solution (X). Known concentrations of the target protein are plotted on the X-axis and the corresponding O.D.<sub>450</sub> on the Y-axis. The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding O.D.<sub>450</sub>. The greater the concentration of target protein in the sample, the higher the O.D.<sub>450</sub>.

*Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.*

### Determine concentration of target protein in unknown sample:

The target protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X-axis at the concentration of antibody in the unknown sample.

### (b) PC Interface Plate Reading

Enter the data into computer program curve fitting software. Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit; avoid using linear regression analysis. Perform a 4-PL regression analysis to calculate the concentration of target analyte in the samples. Multiply the result by the sample dilution factor. See our ELISA data analysis Technical Note on our website, [www.biosensis.com](http://www.biosensis.com) for more instructions on 4\_PL plotting.

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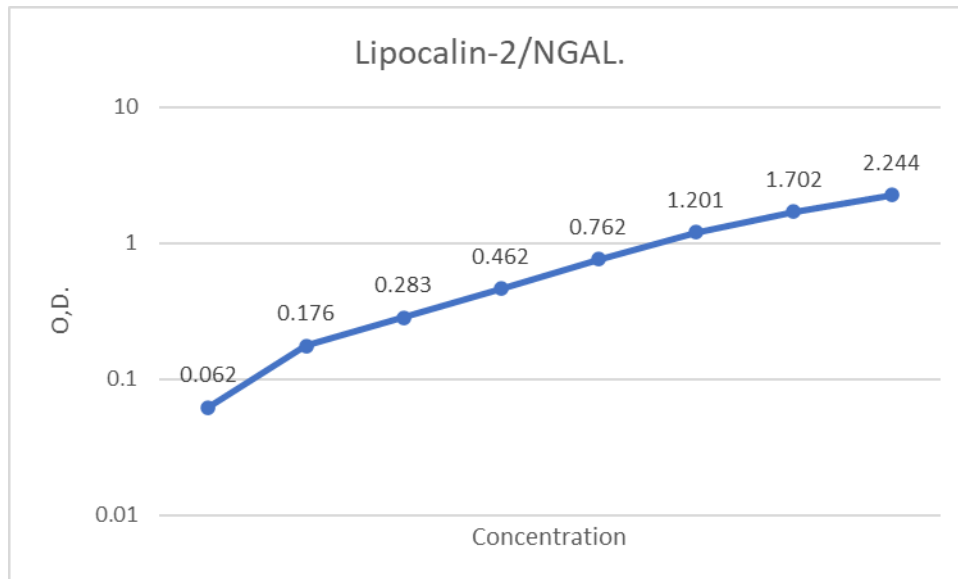
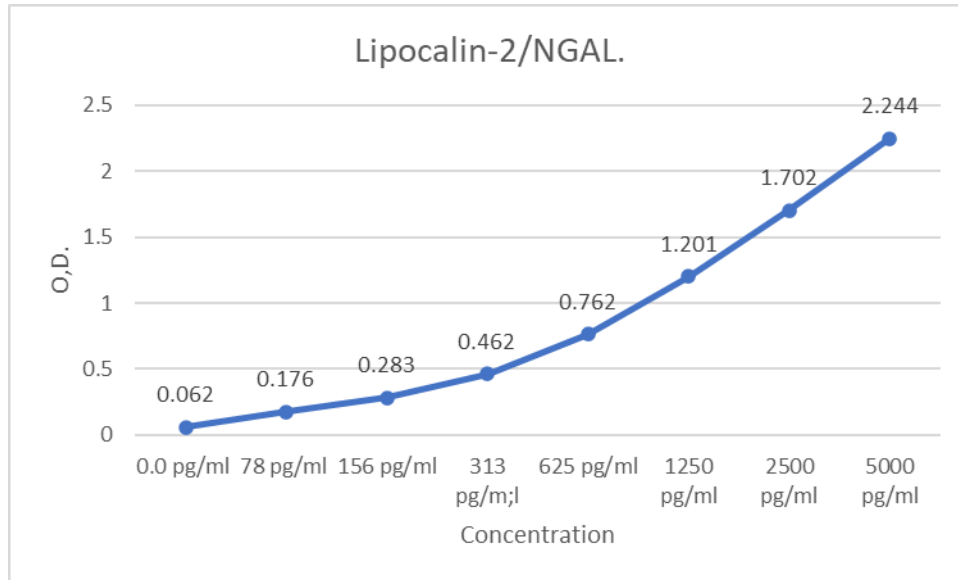
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**IX: Typical Standard Curve (for reference only, not to be used for actual data)**

Concentration pg/ml	0.0 pg/ml	78 pg/ml	156 pg/ml	313 pg/m;l	625 pg/ml	1250 pg/ml	2500 pg/ml	5000 pg/ml
O.D.	0.062	0.176	0.283	0.462	0.762	1.201	1.702	2.244



TMB reaction incubated at 37°C for 25 minute