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Protocol

AccuSignal™ Mouse LCN2 ELISA Kit

This AccuSignal™ high sensitivity sandwich ELISA (Enzyme-Linked Immunosorbent Assay) Kit is an *in vitro* enzyme-linked immunosorbent assay designed for the quantitative detection of Lcn2/Lipocalin-2/NGAL in cell culture supernatants, serum, urine, or plasma (heparin, EDTA).

Parameter	Specification
Range	<78 pg/mL – 5,000 pg/mL
Sensitivity	<10 pg/mL
Specificity	Natural and recombinant Mouse Lcn2
Standard Protein	Expression system for standard: NS0; Immunogen sequence: Q21-N200
Cross-reactivity	There is no detectable cross-reactivity with other relevant proteins.

I. Kit Components

Product	Storage	Size	Item No.
Antibody Coated ELISA Plate	Return unused wells to the foil pouch. Reseal along the entire edge of the zip-seal. May be stored for up to 1 month at 4°C provided this is within the expiration date of the kit.	1 plate	KOA0515A
Target Protein Standard	Discard reconstituted stock solution after 12 hours at 4°C. May be stored at -20°C for 48 hours.	2 vials (10 ng/vial)	KOA0515B
Biotinylated Detection Antibody	May be stored for up to 1 month at 4°C provided this is within the expiration date of the kit.	100 µL (dilution 1:100)	KOA0515C
ABC Complex		100 µL (dilution 1:100)	KAB0101
Sample Diluent		30 mL	KAE0101
Antibody Diluent		12 mL	KAF0101
ABC Diluent		12 mL	KAG0101
Substrate Solution		10 mL	KAC0101
Stop Solution		10 mL	KAD0101
Adhesive Cover		4 covers	-

Note: Kit can be stored 4°C for 6 months, or at -20°C for 12 months from date of manufacture. Avoid multiple freeze-thaw cycles.

II. Additional Materials Required but not Provided

- Microplate reader in standard size
- Automated plate washer
- Adjustable pipettes and pipette tips
- Clean tubes and Eppendorf tubes
- Washing buffer (neutral PBS or TBS).

III. General Considerations

Please read the following instructions before starting the experiment.

- To inspect the validity of experimental operation and the appropriateness of sample dilution proportions, pilot experiment using standards and a small number of samples is recommended.
- The TMB Color Developing agent is colorless and transparent before using, contact us if it is not the case.
- Before using the kit, briefly spin down the vials.
- For statistical reasons, we recommend both standard and samples should be assayed with a minimum of two replicates (duplicates).
- Do not let 96-well plate to dry, this will inactivate active components on plate.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Do not mix or substitute reagents or materials from other kit lots or vendors.
- In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution be prewarmed in 37°C for 30 min before using.

IV. Reagent Preparation

Washing Buffer Preparation

Component	Preparation
0.01 M TBS	• Add 1.2g Tris, 8.5g NaCl; 450 µL of purified acetic acid or 700 µL of concentrated hydrochloric acid to 1000 mL distilled water and adjust pH to 7.2–7.6. • Adjust the total volume to 1 L
0.1 M PBS	• Add 8.5 g sodium chloride, 1.4 g Na_2HPO_4 and 0.2 g NaH_2PO_4 to 1000 mL distilled water and adjust pH to 7.2–7.6. • Adjust the total volume to 1 L

Sample Preparation

Note: Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

- Serum: Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 X g for 15 min. Analyze the serum immediately or aliquot and store samples at -20°C.

- Cell Culture Supernatants: Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20°C.
- Plasma: Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 mins at approximately 1000 x g. Assay immediately or store samples at -20°C.
- Urine: Collect the first urine of the day, micturate directly into a sterile container. Remove impurities by centrifugation, assay immediately or aliquot and store samples at -20°C.

Sample Dilution

Note: Levels of the target protein may vary between different samples. Estimation of the concentration of a target protein in the sample and the optimal dilution factors for each sample must be determined by the investigator. The diluted target protein concentration should fall near the middle of the linear regime in the standard curve. Sample diluent buffer should be used for dilution of samples.

Following are the suggested guidelines for sample dilution. The samples must be mixed well with the diluent buffer.

- High target protein concentration (100000 pg/mL–1000000 pg/mL): The working dilution should be 1:100. For example, add 1 µl sample into a tube with 99 µL sample diluent buffer.
- Medium target protein concentration (10000 pg/mL–100000 pg/mL): The working dilution should be 1:10. For example, add 10 µL sample into a tube with 90µl sample diluent buffer.
- Low target protein concentration (156 pg/mL–10000 pg/mL): The working dilution should be 1:2. For example, add 50 µL sample into a tube with 50µl sample diluent buffer.
- Very Low target protein concentration (0 ng/mL–156 pg/mL): No dilution necessary, or the working dilution should be 1:2.

Reconstitution of the Mouse LCN2 Standard

Note: Mouse Lcn2 standard solution should be prepared no more than 2 hours prior to the experiment. Two vials of Lcn2 standard (10ng/vial) are provided with each kit. Use one vial/experiment.

1. For preparation of 10,000 pg/mL of Mouse Lcn2 standard solution, add 1 mL sample diluent buffer into one vial, keep the vial at room temperature for 10 min and mix thoroughly.
2. Label tubes 1–8.

Tube #	Label
1	5000 pg/mL
2	2500 pg/mL
3	1250 pg/mL
4	625 pg/mL
5	312.5 pg/mL
6	156.25 pg/mL
7	78.13 pg/mL
8	0pg/mL (Blank sample diluent buffer)

3. To generate standard #1, pipette 500µL of reconstituted stock and 500µL of sample diluent into tube #1 and mix thoroughly.
4. Pipette 300µL of the sample diluent buffer into tubes 2–7.
5. Transfer 300µL from tube #1 into tube #2 and mix thoroughly.

6. Transfer 300µL from tube #2 into tube #3 and mix thoroughly, continue further serial dilutions through tube #7.
   Note: The standard solutions are best used within 2 hours. The 10,000 pg/mL standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

Preparation of biotinylated anti-Mouse LCN2 antibody working solution

Note: The solution should be prepared no more than 2 hours prior to the experiment.

- The total volume should be 0.1 mL/well x (the number of wells). Prepare 100–200 µL more of the solution than the total volume required to compensate for pipetting errors.
- Biotinylated anti-Mouse Lcn2 antibody should be diluted 1:100 with the antibody diluent buffer and mixed thoroughly. For example, add 1 µL Biotinylated Anti-Mouse Lcn2 antibody to 99 µL antibody diluent buffer.

Preparation of Avidin-Biotin-Peroxidase Complex (ABC) Working Solution

Note: The solution should be prepared no more than 1 hour prior to the experiment.

- The total volume should be: 0.1 mL/well x (the number of wells). Prepare 0.1–0.2 mL more of the solution than total volume required.
- Avidin-Biotin-Peroxidase Complex (ABC) should be diluted 1:100 with the ABC dilution buffer and mixed thoroughly. For example, add 1 µL ABC to 99 µL ABC diluent buffer.

V. Assay Procedure

Note: ABC working solution and TMB substrate reagent should be warmed at 37°C for 30 min prior to use. Samples and reagents should be mixed thoroughly and evenly after dilution. A standard curve for Lcn2 detection should be prepared for each experiment. It is recommended to determine sample fold dilution by simple estimation of Lcn2 amount in the samples.

1. Aliquot 100 µL per well of each Mouse Lcn2 standard (5000pg/mL, 2500pg/mL, 1250pg/mL, 625pg/mL, 312.5pg/mL, 156.25pg/mL, and 78.13pg/mL) into pre-coated 96-well plate.
2. Add 100 µL of the sample diluent buffer into control wells. Add 100 µL of diluted sample (cell culture supernatants, urine, plasma (EDTA, heparin) or serum into appropriate wells). See “Sample Dilution” above for details. It is recommended to assay all standards, controls and samples in duplicate.
3. Seal the plate with provided adhesive plastic cover and incubate at 37°C for 90 min.
4. Remove the cover and discard the solution. Invert the plate and blot it against clean paper towels or other absorbent material.
   Note: Do NOT let the wells dry completely at any time.
5. Add 100 µL of biotinylated anti-Mouse Lcn2 antibody working solution to each well, seal the plate with adhesive plastic cover and incubate at 37°C for 60 min.
6. Discard the solution and wash the plate 3 times with 0.01M TBS or 0.01M PBS (wash buffer) and each time let washing buffer stay in the wells for ~1 min.
7. Wash by filling each well with 300 µL of wash buffer using a multi-channel pipette or auto washer. Complete removal of liquid at each step is essential to good performance.
   Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling the wells with PBS or TBS buffer.

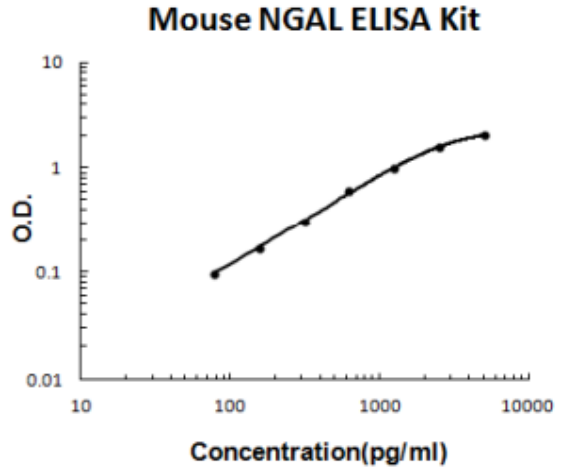
8. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels or other absorbent material.
9. Add 100 µL of prepared ABC working solution to each well, seal the plate and incubate at 37°C for 30 min.
10. Wash plate 5 times with 0.01M TBS or 0.01M PBS as described in step 5.
11. Add 90 µL of TMB substrate reagent to each well, seal the plate and incubate for 25–30 min at 37°C in the dark.
    Note: the optimal incubation time should be determined by the end user.
12. Add 100 µL of stop solution to each well. The color will change to yellow immediately.
13. Read at 450 nm in a microplate reader within 30 min after adding the stop solution.

VI. Calculations

For calculations, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well).

The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The Mouse Lcn2 concentration of the samples can be interpolated from the standard curve.
Note: if the samples measured were diluted, multiply the concentrations obtained from interpolation by the dilution factor.

Kit Specifications



Concentration (pg/mL)	OD
0	0.008
78	0.103
156	0.175
312	0.316
625	0.624
1250	0.992
2500	1.600
5000	2.067

Note: TMB reaction incubation at 37°C for 25–30min. A standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Parameter	Intra-Assay Precision[1]			Inter-Assay Precision[2]		
Sample	1	2	3	1	2	3
N	16	16	16	24	24	24
Mean (pg/mL)	141	785	2588	130	817	2606
Standard Deviation	6.2	38.46	113.87	7.54	54.73	161.57
CV (%)	4.4	4.9	4.4	5.8	6.7	6.2

[1]Precision within an assay: Three samples of known concentration were tested on one plate to assess intra-assay precision.
[2]Precision between assays: Three samples of known concentration were tested in separate assays to assess inter-assay precision.

Troubleshooting

Visit www.rockland.com/ELISA-kit-troubleshooting

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