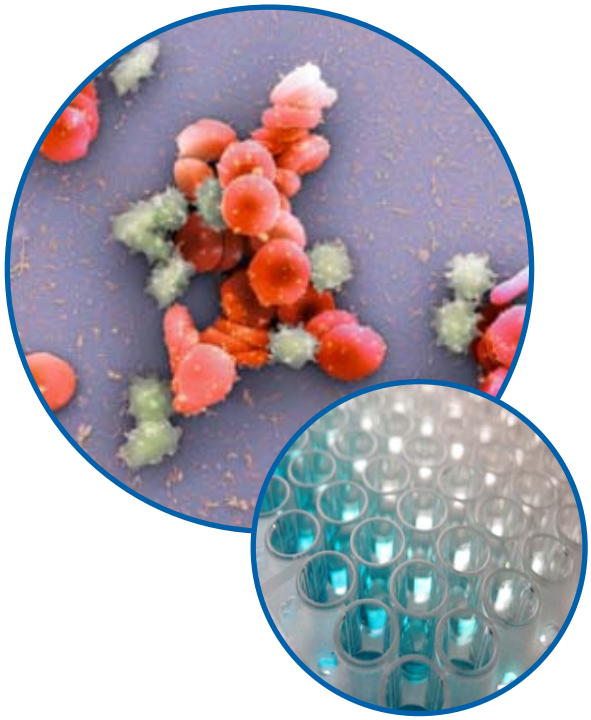


AdipoGen® LIFE SCIENCES

MANUAL

Lipocalin-2 (mouse) ELISA Kit

[NGAL (mouse) ELISA Kit]

For research use only. Not for diagnostic use

Version 1 (May-06-2015)

Cat. No. AG-45B-5004-KI01

Table of Contents

1. Intended Use

The Lipocalin-2 (mouse) ELISA Kit is to be used for the *in vitro* quantitative determination of mouse Lipocalin-2 in cell culture supernatants, serum and plasma. This ELISA Kit is for research use only.

2. Introduction

Lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL), 24p3, or neutrophil lipocalin (NL), is a 25-kDa secretory glycoprotein (1).

Lipocalin 2 was initially defined as a powerful bacteriostatic agent active against various Gram-negative microorganisms through impeding bacterial iron sequestration (2, 3). LCN2 also functions as a stress protein that is released in a variety of other sterile inflammatory conditions such as adipose obesity-related inflammation and cancer (2, 3).

LCN2 is over-expressed in many types of cancers including breast, pancreatic, and ovarian carcinomas and promotes tumorigenesis through enhancing tumor cell survival and proliferation, and metastatic potential (4, 5, 6).

Furthermore, a growing body of evidence suggests that serum levels of lipocalin-2 are correlated with obesity, insulin resistance, hyperglycemia, coronary heart disease, acute renal injury and fatty liver disease (7, 8, 9).

3. General References

(1) Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase: L. Kjeldsen, et al.; *J. Biol. Chem.* **268,** 10425 (1993)

(2) An iron delivery pathway mediated by a lipocalin: J. Yang, et al.; *Mol. Cell* **10,** 1045 (2002)

(3) Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron: T.H. Flo, et al.; *Nature* **432,** 917 (2004)

(4) Lipocalin 2 promotes breast cancer progression: J. Yang; *PNAS* **106,** 3913 (2009)

(5) Lipocalin 2 diminishes invasiveness and metastasis of Ras-transformed cells: J. Hanai, et al.; *J. Biol. Chem.* **280,** 13641 (2005)

(6) Ectopic expression of neutrophil gelatinase-associated lipocalin suppresses the invasion and liver metastasis of colon cancer cells: H.J. Lee, et al.; *Int. J. Cancer* **118,** 2490 (2006)

(7) Lipocalin-2 is an inflammatory marker closely associated with obesity, insulin resistance, and hyperglycemia in humans: Y. Wang, et al.; *Clin. Chem.* **53,** 34 (2007)

(8) The adipokine lipocalin 2 is regulated by obesity and promotes insulin resistance: Q.W. Yan, et al.; *Diabetes* **56,** 2533 (2007)

(9) Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery: J. Mishra, et al.; *Lancet* **365,** 1231 (2005)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of mouse Lipocalin-2 in cell culture supernatants, serum and plasma. A polyclonal antibody specific for Lipocalin-2 has been precoated onto the 96-well microtiter plate. Standards (STD) and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, Lipocalin-2 is recognized by the addition of a biotinylated polyclonal antibody specific for mouse Lipocalin-2 (DET). After removal of excess biotinylated antibody, streptavidin-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of mouse Lipocalin-2 in the samples.

5. Handling & Storage

- Reagents must be stored at 2-8°C when not in use.
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

• 1 vial mouse Lipocalin-2 Standard (lyophilized)	(10 ng)	(STD)
• 1 vial Detection Antibody (100x)	(120 µl)	(DET)
• 1 vial HRP Labeled Streptavidin (200x)	(60 µl)	(STREP-HRP)
• 2 bottles Wash Buffer 10X	(2 x 25 ml)	(Wash Buffer 10X)
• 1 bottle Assay Buffer 5X	(20 ml)	(Assay Buffer 5X)
• 1 bottle TMB Substrate Solution	(12 ml)	(TMB)
• 1 bottle Stop Solution	(12 ml)	(STOP)
• 1 plate coated with Lipocalin-2 Antibody	(12 x 8-well strips)	
• 2 plate Covers (plastic film)		
• 2 silica Gel Minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450nm
- Calibrated precision pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 50 ml Wash Buffer 10X + 450 ml distilled or deionized water) to obtain Wash Buffer 1X. The 1x Wash buffer may be stored at 2-8°C for up to one month.

- **Assay Buffer 5X** has to be diluted with deionized water 1:5 before use (e.g. 10 ml Assay Buffer 5X + 40 ml distilled or deionized water) to obtain Assay Buffer 1X. The 1x Assay buffer may be stored at 2-8°C for up to one month.

- **Detection Antibody (DET) 100x** (after a spin-down) has to be diluted to 1:100 in Assay Buffer 1X (20 µl DET + 1.98 ml Assay Buffer 1X). 100µl of the 1x Detection antibody is required per well.

  NOTE: *Prepare only as much 1x Detection antibody as needed. The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP Labeled Streptavidin (STREP-HRP) 200X** (after a spin-down) has to be diluted to 1:200 with Assay Buffer 1X (50 µl in 9.95 ml of Assay Buffer 1X). 100µl of the 1x STREP-HRP is required per well.

  NOTE: *Prepare only as much 1x STREP-HRP as needed. The diluted STREP-HRP is not stable and cannot be stored!*

- **Mouse Lipocalin-2 Standard (STD)** has to be reconstituted with 1ml of Assay Buffer 1X.

  - This reconstitution produces a stock solution of 10 ng/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes **at RT.** Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C!*

  - Dilute the standard protein concentrate (STD) in Assay Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in Assay Buffer 1X is recommended.

  - Suggested standard points are:

    10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 and 0 ng/ml.

To obtain	**Add**	Into
10 ng/ml	10 ng/ml stock	**-**
5 ng/ml	250 µl of Lipocalin-2 (10 ng/ml)	250 µl of Assay buffer 1X
2.5 ng/ml	250 µl of Lipocalin-2 (5 ng/ml)	250 µl of Assay buffer 1X
1.25 ng/ml	250 µl of Lipocalin-2 (2.5 ng/ml)	250 µl of Assay buffer 1X
0.625 ng/ml	250 µl of Lipocalin-2 (1.25 ng/ml)	250 µl of Assay buffer 1X
0.312 ng/ml	250 µl of Lipocalin-2 (0.625 ng/ml)	250 µl of Assay buffer 1X
0.156 ng/ml	250 µl of Lipocalin-2 (0.312 ng/ml)	250 µl of Assay buffer 1X
0 ng/ml	250 µl of Assay buffer 1X	Empty tube

NOTE: *Do not store the diluted standard solutions.*

8.2. Sample Collection, Storage and Dilution

Cell Culture Supernatants, serum and plasma have to be diluted in Assay Buffer 1X. Starting dilutions of 1/40 to 1/80 are recommended.

8.3. Assay Procedure (Checklist)

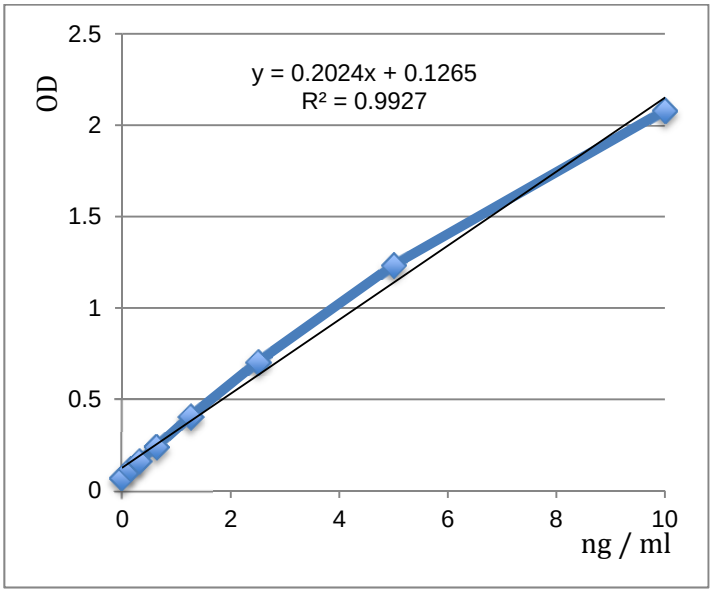
☐	1. Determine the number of 8-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the bag with 2 silica gel minibags and stored at 4°C. **NOTE:** *Remaining 8-well strips coated with* Lipocalin-2 *antibody when opened can be stored in the presence of 2 silica gel minibags at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted serum, plasma or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2 Preparation of Samples).**
☐	3. Cover the plate with plastic film and incubate for **1 h at room temperature.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the 1x Detection Antibody **(DET) (see 8.1 Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plastic film and incubate for **1 h at room temperature.**
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(STREP-HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plastic film and incubate for **20 min at room temperature**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB substrate solution **(TMB)**.
☐	12. Allow the color reaction to develop **at room temperature in the dark for 15 minutes**. Do not cover the plate.
☐	13. Stop the reaction by adding 100 µl of Stop Solution (**STOP**). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (**STOP**) is added.
	! CAUTION: CORROSIVE SOLUTION !
☐	14. Measure the OD at 450 nm in an ELISA reader.

9. Calculation of Results

- Average the duplicate readings for each standard, control and sample and subtract the average blank value (obtained with the 0 pg/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding Lipocalin-2 concentration (pg/ml) on the vertical axis (see **10.** TYPICAL DATA).
- Calculate the Lipocalin-2 concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation
- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate the concentration of mouse Lipocalin-2 in the sample.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:



Mouse Lipocalin-2 (ng/ml)	Absorbance (450nm)
0	0.074
0.156	0.122
0.312	0.162
0.625	0.239
1.25	0.406
2.5	0.707
5	1.236
10	2.082

Figure: Standard curve

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of mouse Lipocalin-2 that can be detected by this assay is 0.1 ng/ml. **NOTE:** *The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.156 ng/ml – 10 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant mouse Lipocalin-2**.** It does not cross-react with human Lipocalin-2.

D. Intra-inter assay precision:

Intra-assay Precision (Precision within an assay)	C.V	<7%
Inter-assay Precision (Precision between assays)	C.V	<12%

E. Expected values:

Mouse Lipocalin-2 levels range in plasma or serum from **<50 ng /ml to >1 µg /ml**.

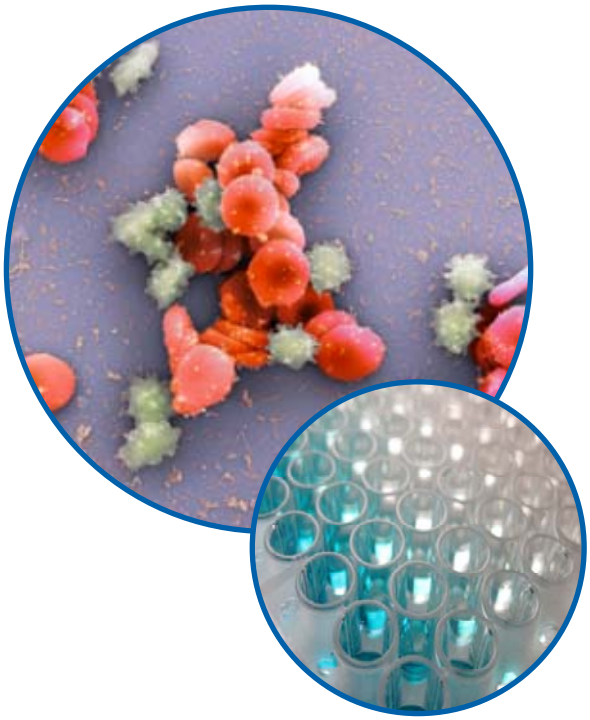
12. Technical Hints and Limitations

- It is recommended that all standards, controls and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 8-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Solution protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of STREP-HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Notes



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