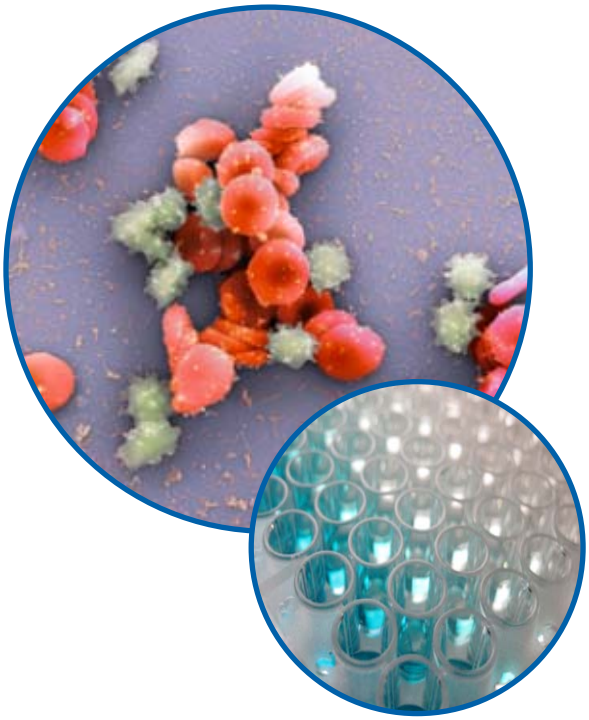


AdipoGen® LIFE SCIENCES

MANUAL

Nampt (Visfatin/PBEF) (human) ELISA Kit

For research use only. Not for diagnostic use.

Version 3 (04-May-2015)

Cat. No. AG-45A-0006YEK-KI01

Table of Contents

1. Intended Use

The Nampt (Visfatin/PBEF) (human) ELISA Kit is to be used for the *in vitro* quantitative determination of human Nampt in serum. This ELISA Kit is for research use only.

2. Introduction

Fukuhara et al. (1) isolated visfatin, an adipocytokine that is highly enriched in the visceral fat of both humans and mice and whose expression level in plasma increases during the development of obesity. Visfatin corresponds to pre-B cell colony-enhancing factor (PBEF), a 52-kD cytokine expressed in lymphocytes. The gene encoding PBEF was originally isolated from an activated lymphocyte cDNA library (2). Although PBEF lacks a typical signal sequence for secretion, transfected COS-7 and mouse embryonic fibroblasts secreted PBEF into the culture medium. Samal et al. (2) found that recombinant PBEF secreted from transfected COS-7 and mouse embryonic fibroblasts was not itself active in a pre-B-cell colony formation assay, but it synergized the pre-B-cell colony formation activity of stem cell factor and interleukin-7. Jia et al. (3) found that PBEF is an inflammatory cytokine that plays a requisite role in the delayed neutrophil apoptosis of sepsis. Visfatin exerted insulin-mimetic effects in cultured cells and lowered plasma glucose levels in mice. Mice heterozygous for a targeted mutation in the visfatin gene had modestly higher levels of plasma glucose relative to wild type littermates. Surprisingly, it was found that visfatin binds to and activates the insulin receptor (1). However, this original discovery has not been reproduced by two groups (4-5). Visfatin, which is a secretory form of Nampt (nicotinamide phosphoribosyl-transferase), the rate-limiting enzyme of the mammalian NAD, plays a key role in secretion of insulin in the pancreatic beta-cells (5). Recently, two recent studies showed that plasma or serum levels of visfatin in patients with type 2 diabetes mellitus was elevated (6-7), suggesting that measurement of plasma visfatin provides a relevant tool for understanding metabolic diseases.

3. General References

(1) Visfatin: a protein secreted by visceral fat that mimics the effects of insulin: A. Fukuhara, et al.; Science **307,** 426 (2005)

(2) Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor: B. Samal, et al.; Mol. Cell. Biol. **14,** 1431 (1994)

(3) Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis: S.H. Jia, et al.; J. Clin. Invest. **113,** 1318 (2004)

(4) Molecular characteristics of serum visfatin and differential detection by immunoassays: A. Körner, et al.; J. Clin. Endocrinol. Metab. **92,** 4783 (2007)

(5) Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme: J.R. Revollo, et al.; Cell Metab. **6,** 363 (2007)

(6) Correlation of circulating full-length visfatin (PBEF/Nampt) with metabolic parameters in subjects with and without diabetes: a cross-sectional study: R. Retnakaran, et al.; Clin. Endocrinol. **69,** 885 (2008)

(7) Elevated Plasma Level of Visfatin/Pre-B Cell Colony-Enhancing Factor in Patients with Type 2 Diabetes Mellitus: M.P. Chen, et al.; J. Clin. Endocrinol. Metab. **91,** 28 (2006)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human Nampt in biological fluids. A monoclonal antibody specific for Nampt has been precoated onto the 96-well microtiter plate. Standards and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, Nampt is recognized by the addition of a purified polyclonal antibody specific for Nampt (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-rabbit IgG (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 Nampt in the samples.

5. Handling & Storage

- Reagent 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 plate coated with human Nampt Antibody	(6 x 16-well strips)	
2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
2 bottles ELISA Buffer 10X	(2 x 30 ml)	(ELISA Buffer 10X)
1 vial Detection Antibody	(60µl)	(DET)
1 vial HRP 100X (HRP Conjugated anti-rabbit IgG)	(150 µl)	(HRP 100X)
1 vial human Nampt Standard (lyophilized)	(16 ng)	(STD)
1 bottle TMB Substrate Solution	(12 ml)	(TMB)
1 bottle Stop Solution	(12 ml)	(STOP)
2 plate sealers (plastic film)		
2 silica Gel Minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450 nm
- Calibrated precision single and multi-channel 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 water) to obtain Wash Buffer 1X.

- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 20 ml ELISA Buffer 10X + 180 ml water) to obtain ELISA Buffer 1X.

- **Detection Antibody (DET)** has to be diluted to 1:200 in ELISA Buffer 1X
  (50 µl DET + 10 ml ELISA Buffer 1X).
  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP 100X (HRP Conjugated anti-rabbit IgG)** has to be diluted to the working concentration by adding 100 µl in 10 ml of ELISA Buffer 1X (1:100).
  NOTE: *The diluted HRP is used within one hour of preparation.*

- **Human Nampt Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 16 ng/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

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

  - Dilute the standard protein concentrate (STD) (**16 ng/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.

  - Suggested standard points are:

    8 , 4 , 2 , 1 , 0.5 , 0.25 , 0.125 and 0 ng/ml.

Dilute further for the standard curve:

To obtain	Add	Into
8 ng/ml	300 µl of Nampt (16 ng/ml)	300 µl of ELISA Buffer 1X
4 ng/ml	300 µl of Nampt (8 ng/ml)	300 µl of ELISA Buffer 1X
2 ng/ml	300 µl of Nampt (4 ng/ml)	300 µl of ELISA Buffer 1X
1 ng/ml	300 µl of Nampt (2 ng/ml)	300 µl of ELISA Buffer 1X
0.5 ng/ml	300 µl of Nampt (1 ng/ml)	300 µl of ELISA Buffer 1X
0.25 ng/ml	300 µl of Nampt (0.5 ng/ml)	300 µl of ELISA Buffer 1X
0.125 ng/ml	300 µl of Nampt (0.25 ng/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 1X	Empty tube

8.2. Sample Collection, storage and dilution

Serum : Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

Serum has to be used undiluted. Samples containing visible precipitates must be clarified before use.

NOTE: *If sample values fall outside the detection range of the assay, a lower or higher dilution in ELISA Buffer 1X may be required!*

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 16-well strips coated with Nampt antibody when opened can be stored 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 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 plate sealer and incubate **overnight at 4°C.**
☐	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 three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the Detection Antibody **(DET) (see 8.1. Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	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 three 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 Conjugated anti-rabbit IgG) **(HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	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 (RT°C) in the dark for 10 minutes**.
☐	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 is added.
	! CAUTION: CORROSIVE SOLUTION!
☐	14. Measure the OD at 450 nm in an ELISA reader within 30 minutes.

9. Calculation of Results

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

10. Typical Data

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

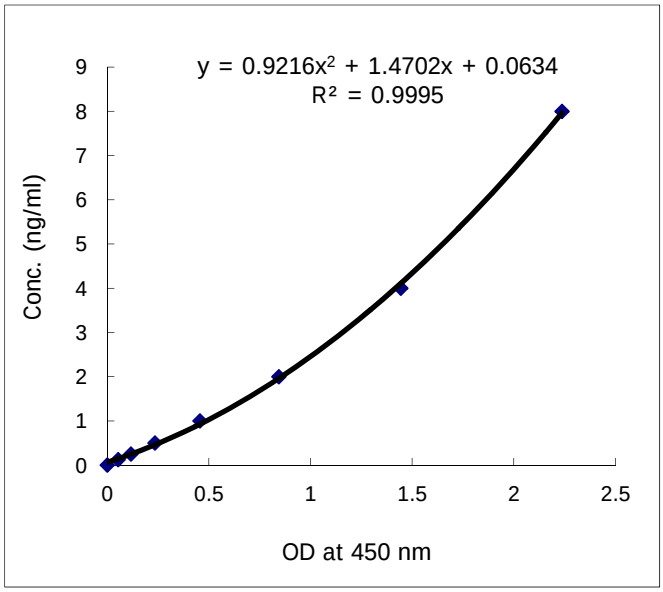


Figure: Standard curve

Standard hNampt (ng/ml)	Optical Density (mean)
8	2.237
4	1.444
2	0.844
1	0.456
0.5	0.235
0.25	0.117
0.125	0.054
0	0

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of Nampt that can be detected by this assay is 30 pg/ml. ***NOTE:*** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.125 ng/ml – 8 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human Nampt. It does not cross-react with human adiponectin, human resistin, human vaspin, human RBP4, human GPX3, human progranulin, human IL-33, human clusterin, human ANGPTL3, human ANGPTL4, human ANGPTL6, mouse RBP4.

Mouse Nampt shows weakly 5% cross-reactivity in this assay.

Rat Nampt shows weakly 15% cross-reactivity in this assay.

D. Intra-assay precision:

Four samples of known concentrations of human Nampt were assayed in replicates 4 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	1.75	0.04	2.31	4
2	2.87	0.16	5.58	4
3	1.51	0.08	5.53	4
4	1.48	0.14	9.11	4

E. Inter-assay precision:

Four samples of known concentrations of human Nampt were assayed in 7 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	2.92	0.18	6.24	7
2	1.02	0.07	7.24	7
3	0.72	0.03	4.66	7
4	1.01	0.06	5.56	7

F. Recovery:

When samples (serum) are spiked with known concentrations of human Nampt, the recovery averages 98% (range from 90% to 110%).

Samples	Average recovery (%)	Range (%)
1	101.41	95-105
2	91.77	90-100
3	100.16	95-105
4	99.13	95-105

G. Linearity:

Different human serum samples containing Nampt were diluted several fold (1 to 1/4) and the measured recoveries ranged from 85% to 105%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
1	1	1.89	1.89	100
	1 : 2	0.95	0.98	103.59
	1 : 4	0.47	0.46	97.58
2	1	0.66	0.66	100
	1 : 2	0.33	0.34	102.60
	1 : 4	0.16	0.16	97.76
3	1	1.39	1.39	100
	1 : 2	0.69	0.65	93.93
	1 : 4	0.35	0.30	86.20

H. Expected values:

Nampt levels range in serum from **0.2 to > 1.5 ng/ml** (from healthy donors).

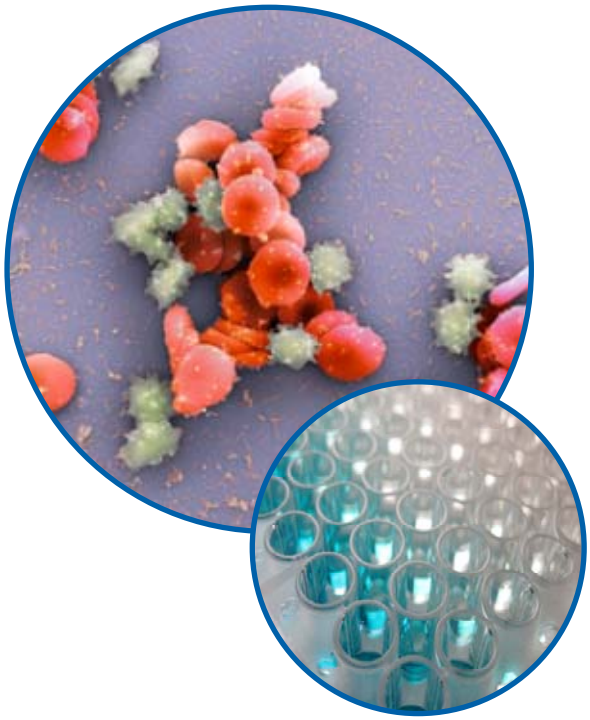
12. Technical Hints and Limitations

- 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 100 µl 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 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Substrate Solution (TMB) protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution (STOP) 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 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



Product Specific References:

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For more References please visit www.adipogen.com!

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