Product Manual

Nampt (Visfatin/PBEF) (human) Intracellular ELISA Kit

Catalog #: AG-45A-0006EK

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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INTENDED USE

The Nampt (Visfatin/PBEF) (human) Intracellular ELISA Kit is to be used for the *in vitro* quantitative measurement of natural and recombinant human Nampt in cell lysates. This ELISA Kit is for research use only.

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 phosphoribosyltransferase), 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.

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 colonyenhancing 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)

ASSAY PRINCIPLE

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human Nampt incell lysates. A monoclonal antibody specific for Nampt has been pre-coated 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 Detector is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3’,5,5’-tetramethylbenzidine (TMB). The Stop Solution is then added to stop the reaction (following 10 minutes of color development in the dark) resulting in a shift from blue to a yellow product in the plate. 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.

Reagents require separate storage conditions.

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.

MATERIALS SUPPLIED

- 1 plate coated with human Nampt Antibody, (12 x 8-well strips)
- 1 bottle Wash Buffer 10X, (2x30ml)
- 1 bottle Diluent 10X, (2x30ml)
- 1 bottle Detection Antibody, (60µl)
- 1 vial Detector 100X, (150µl)
- 1 vial human Nampt Standard (lyophilized), (32 ng)
- 1 bottle Lysis Buffer 10X (12ml),
- 1 bottle TMB Substrate Solution, (12ml)
- 1 bottle Stop Solution, (12ml)
- 2 plate sealers (plastic film),

ADDITIONAL MATERIALS AND EQUIPMENT NEEDED

- Microtiterplate reader at 450nm 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

ASSAY PROTOCOL

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. 50ml Wash Buffer 10X + 450ml water) to obtain Wash Buffer 1X.

Diluent 5X has to be diluted with deionized water 1:5 before use (e.g. 50ml Diluent 5X + 200ml water) to obtain Diluent 1X.

Detection Antibody Warm Detection Antibody to room temperature. Dilute the antibody 1:250 in 1X Diluent (8 µl antibody + 1992 µl 1X Diluent). Diluted antibody cannot be stored.

Detector 100X Dilute 100X Detector 1:99 with 1X Diluent (100 µl Detector + 9.9 ml of 1X Diluent). **NOTE:** The diluted Detector is used within one hour of preparation.

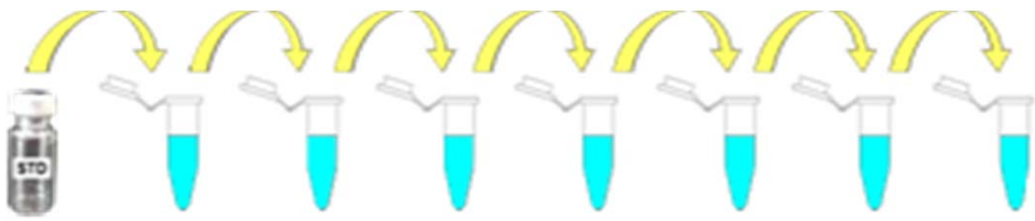
Human Nampt Standard (STD) has to be reconstituted with 1ml of deionized water.

Storage Notes

- This reconstitution produces a stock solution of 32 ng/ml. Mix the standard stock solution to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. **NOTE:** The reconstituted standard is aliquoted and stored at -20°C.
- Dilute the standard protein concentrate (STD) (32 ng/ml) in Diluent 1X. A seven-point standard curve using 2-fold serial dilutions in Diluent 1X is recommended.
- Standard points are 16, 8, 4, 2, 1, 0.5, 0.25, and 0 ng/ml. **Follow standard curve preparation chart and diagram for specific instructions regarding standard curve preparation.**

Dilute further for the standard curve:

To obtain	Add	Into
16 ng/ml	300 µl of Nampt (32 ng/ml)	300 µl of 1X Diluent
8 ng/ml	300 µl of Nampt (16 ng/ml)	300 µl of 1X Diluent
4 ng/ml	300 µl of Nampt (8 ng/ml)	300 µl of 1X Diluent
2 ng/ml	300 µl of Nampt (4 ng/ml)	300 µl of 1X Diluent
1 ng/ml	300 µl of Nampt (2 ng/ml)	300 µl of 1X Diluent
0.5 ng/ml	300 µl of Nampt (1 ng/ml)	300 µl of 1X Diluent
0.25 ng/ml	300 µl of Nampt (0.5 ng/ml)	300 µl of 1X Diluent
0 ng/ml	300 µl of 1X Diluent	Empty tube



SAMPLE COLLECTION, STORAGE AND DILUTION

Cells Lysates: Ice-cold 1X Lysis Buffer and 1X Diluent are prepared by 1:9 dilutions with dH2O and placed on ice until needed. Grow cells to 80-90 % confluency. Adherent cells can be scraped off plate and transferred to a tube; suspension cells pipetted to appropriate tube. Centrifuge at 700-1000 x g for 5 min at 4°C and carefully remove and discard supernatant. Wash 1-2 times with ice-cold PBS. Add 200 µl ice-cold 1X Lysis Buffer with 1 mM PMSF (not included) per 1 x 107 cells and allow to stand on ice for 30 min. Centrifuge at 10K x g for 5 min at 4°C and transfer supernatant to a new tube. The supernatant is the cell lysate and should be freshly prepared and diluted into 1X Diluent. As a starting point 1/10 to 1/1000 dilutions are recommended. If samples fall outside the assay range a lower or higher dilution may be required.

***NOTE**: If samples fall the outside range of assay, a lower or higher dilution may be required.*

ASSAY PROCEDURE (CHECKLIST)

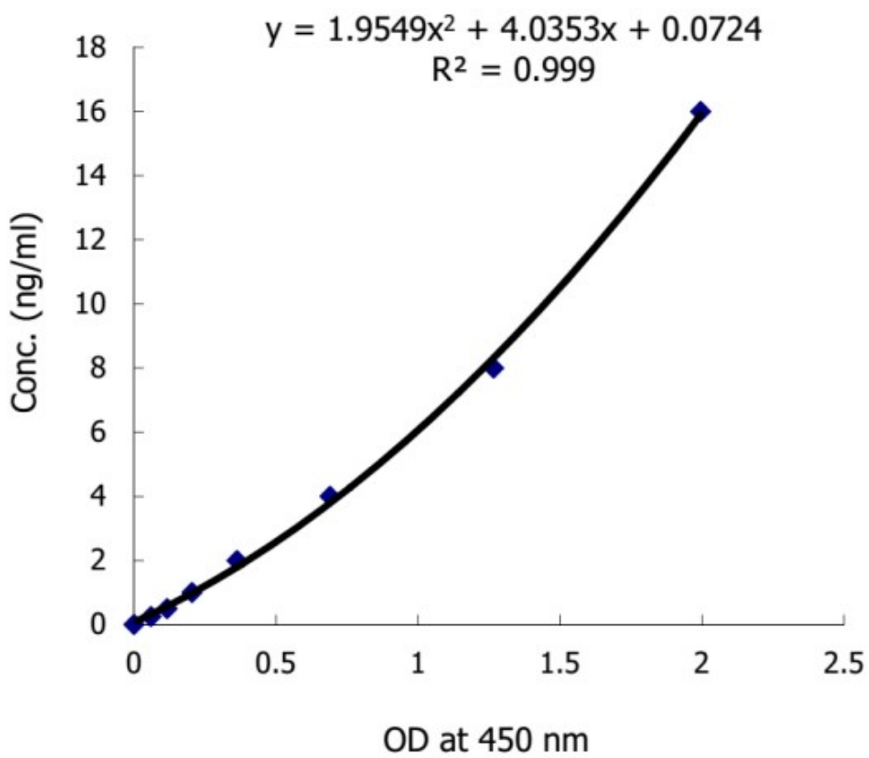
☐	1.	Determine the number of -well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C.
***NOTE**: Remaining well strips coated with Nampt antibody when opened can be stored at 4°C for up to 1 month.*		
☐	2.	Add 100µl of the different standards into the appropriate wells in duplicate! At the same time, add 100µl of undiluted serum samples in duplicate to the wells (**see Preparation and Storage of Reagents and Preparation of Samples**).
☐	3.	Cover the plate with plate sealer and incubate for overnight at 4°C.
☐	4.	Aspirate the coated wells and add 300µl 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 100µl to each well of the Detection Antibody (**see 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 300µl 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 100µl to each well of the diluted 1X Detector (**see 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 300µl 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 100µl to each well of TMB Substrate Solution.
☐	12.	Allow the color reaction to develop at room temperature (RT°C) in the dark for 10 minutes.
☐	13.	Stop the reaction by adding 100µl of Stop Solution. 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.

CALCULATION OF RESULTS

- Average the duplicate readings for each standard, QC 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 TYPICAL STANDARD CURVE GRAPH).
- 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.

TYPICAL STANDARD CURVES

Typical standard curves are shown below. These curves must not be used to calculate hNampt concentrations; each user must run a standard curve for each assay.



PERFORMANCE CHARACTERISTICS

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.*

Assay range

0.25 ng/ml – 16 ng/ml

This ELISA is specific for the measurement of natural and recombinant human Nampt. It does not cross-react with human adiponectin, human resistin, human RELM-β, human leptin, human GPX3, human ANGPTL4, human FABP4, human ANGPTL6, human PAI1. **Mouse Nampt shows weak cross-reactivity in this assay (5 %). Rat Nampt shows weak cross-reactivity in this assay (15 %).**

Intra-assay precision

Two samples of known concentration were assayed in replicates of 10 in order to test precision within an assay.

Samples	Mean (ng/ml)	SD	CV (%)	n
# 1	244.46	6.66	2.73	10
# 2	248.13	24.21	9.76	10

Inter-assay precision

Two samples of known concentration were assayed in 6 separate assays to test precision between assays.

Samples	Mean (ng/ml)	SD	CV (%)	n
# 1	209.59	8.42	4.02	6
# 2	251.04	18.58	7.40	6

SAMPLE RECOVERIES

Human cell lysates were spiked with known concentrations of human Nampt. The recovery averages were 98% (range from 90% to 105%.

Samples	Average Recovery (%)	Range (%)
# 1	96.36	95 - 105
# 2	102.62	95 - 105

NOTE: *Mouse Nampt shows weak cross-reactivity in this assay (5 %). Rat Nampt shows weak cross-reactivity in this assay (15 %).*

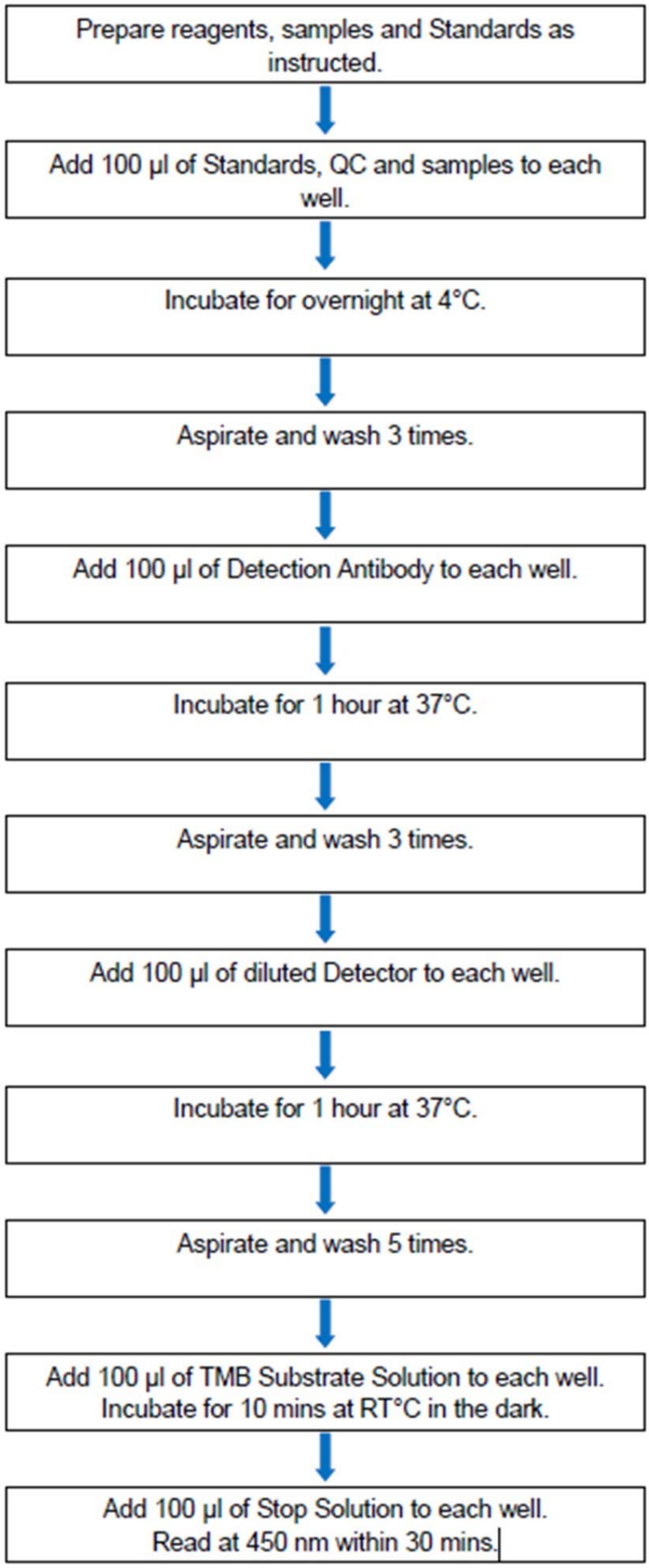
TECHNICAL HINTS AND LIMITATIONS

- It is recommended that all standards, QC samples 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 8-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Substrate Solution protected from light.
- The Stop Solution consists of phosphoric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

TROUBLESHOOTING

Problem	Possible Causes	Solutions
No signal or weak signal	Omission of key reagent	Check that all reagents have been added
	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 detector 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
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 doublecheck calculations.

ASSAY FLOW CHART



PRODUCT SPECIFIC REFERENCES

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